'DRIPPY GILL' - AN OOZE DISEASE OF THE CULTIVATED MUSHROOM *AGARICUS BISPORUS* CAUSED BY *PSEUDOMONAS AGARICI*

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ABBREVIATIONS

ANOVA	analysis of variance
ATCC	American Type Culture Collection
CANU-PMS	Culture collection of the Department of Plant and Microbial Sciences, University of Canterbury
cfu	colony forming units
CMM	compost malt medium
HBH	4-hydroxybenzoyl hydrazine
ICMP	International Collection of Micro-organisms from Plants
KA	King's medium A
KB	King's medium B
NA	nutrient agar
NCPPB	National Collection of Plant Pathogenic Bacteria, England
NB	nutrient broth
PBS	phosphate buffered saline
PEG	polyethylene glycol
RH	relative humidity
TCH	thiocarbohydrazide
TEM	transmission electron microscope

ABSTRACT

'Drippy gill', a serious bacterial disease of cultivated mushrooms, was investigated. The causal organism, *Pseudomonas agarici*, causes profuse ooze from the gills of affected sporocarps and intrahyphal transmission was suspected. However, the majority of bacteria were shown to occur extrahyphally with only occasional intrahyphal presence. Enzymatic and developed gold labelling techniques showed *P. agarici* was essentially unable to actively penetrate fungal hyphae.

In the presence of *P. agarici* cells, the extrahyphal matrix was actively degraded as were the outer fungal wall layers. *P. agarici* was shown not to produce chitinase, however, ß-glucanase activity was evidenced by dissolution of the extracellular matrix and glucan of the *Agaricus* hyphal wall.

Unlike most other fluorescent pseudomonads of the mushroom casing layer, both *P. agarici* and PV29 did not readily show transformation of the smooth wild type colony form to the rough variant.

The unique features of the 'drippy gill' organism such as lack of toxin production and the ability to penetrate *Agaricus* protective membranes are discussed and an epidemiology of 'drippy gill' disease forwarded.

A new mushroom disease, 'cavity-cap', was recorded and the causal organism identified as *P. cepacia* (later re-classified as *P. gladioli* var *agaricicola*).

CHAPTER ONE

GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

Mushroom growers are constantly battling against a number of factors, both biotic and abiotic, which contrive to prevent the grower from achieving a maximum yield of marketable quality mushrooms. In modern cropping systems, abiotic factors (such as temperature, humidity and substrate water content) are largely controlable. The biotic factors, however, frequently prove to be less than manageable and their influence is often devastating to the crop. The abiotic factors are not entirely blameless for this situation. The mesophilic, humid conditions which prevail are ideal for growing mushrooms but unfortunately, they are also optimal or near optimal for other mesophilic organisms, from viruses through to flies, and their larvae. Principal components of mushroom compost and casing (straw, animal manure, peat-soil, lime and water), are imported from sources outside the mushroom farm and consequently opportunities for contamination and infection by deleterious organisms are numerous and vehicles for disease transmission are many. Of the biotic factors regarded as detrimental to mushroom crops (Table 1.1) (Fletcher *et al* 1989), it is the bacteria which are the focus of this study.

1.2 Bacteria in Mushroom Culture

Bacteria play an essential role in all facets of mushroom production. They are crucial in composting, initiate sporocarp production and limit the quality and value of the final product. Within the compost, bacteria make available many of the nutrients required by mushroom mycelium and they are also a significant contributor to the compost biomass (Fletcher 1979). Of the bacterial microflora of the casing layer, over half are pseudomonads, approximately 47% of which are a single species, *Pseudomonas putida* (Samson *et al* 1987). Fruiting potentiators - bacteria which stimulate rather than inhibit mushroom development - are well known. The first record of such a bacterium, *Bacillus psilocybe*, was made in 1956 by Urayama (Stamets and Chilton 1983).

PEST	TARGET[a]	MODE OF ACTION	SYMPTOM	EFFECT ON CROP
INSECTS	M & S	Direct feeding and burrowing into sporocarp	Colonization by larvae and subsequent bacterial rot	Yield reduction
MITES	M & S	Direct feeding	Discolouration	Yield reduction
NEMATODES	M	Direct feeding	-	Yield reduction
PARASITIC FUNGI	M & S	Parasitism	Decay, distortion and discolouration	Serious crop loss
ANTAGONISTIC FUNGI	M & S	Competition and prod. of volatiles	Distortion and discolouration	Yield reduction
BACTERIA	M & S	Toxin production and soft rots	Decay, distortion and discolouration	Serious crop loss
VIRUSES	M & S	Intracellular invasion	Distortion and abnormalities	Yield reduction

[a] M = Mycelium; S = Sporocarp

Table 1.1 The Biotic Pests of Mushroom Crops

Hayes *et al* (1969) isolated and identified *P. putida* as an important potentiator and this pseudomonad was the subject of a proposed model explaining their stimulatory effect on sporocarp initiation and development by Rainey and Cole (1987) and Rainey (1989).

The casing layer is also a source of pathogens of which the bacteria are highly significant especially *Pseudomonas* spp. (Fletcher 1979). Diseases of mushrooms caused by fluorescent pseudomonads are well documented. *P. tolaasii* Paine and *P. gingeri* Preece and Wong are the cause of brown blotch (Paine 1919) and ginger blotch (Wong *et al* 1982) respectively. 'Drippy gill' disease is caused by *P. agarici* Young (Young 1970) and mummy disease has also been associated with a fluorescent pseudomonad (Schisler *et al* 1968; Betterley and Olson 1987). A disease similar to 'drippy gill' was reported to be caused by another fluorescent pseudomonad, designated PV29, by Rainey and Cole (1988).
P. tolaasii, the causal organism of mushroom brown blotch, is a particularly virulent bacterium which may result in losses of mushroom crops up to 10% and a further 10% due to loss in quality and market value (Fermor *et al* 1991) Distribution of this bacterium is reportedly confined to cultured Basidiomycetes (Bessette 1984) and is introduced to the mushroom beds via the peat and limestone of the casing material (Wong and Preece 1980). Once established, it spreads through the cropping shed and mushroom farm by flies, mites, dust particles, spores of infected mushrooms and pickers and their tools (Wong and Preece 1980) and may go on to infect other crops.

1.3 The Fluorescent Pseudomonads

The majority of disease causing bacteria isolated from sporocarps and mushroom growing beds are representatives of the genus *Pseudomonas*. All these, with one exception, *Pseudomonas gladiolii* pv *agaricicola* (Lincoln *et al* 1991; Gill and Cole 1992 as *P. cepacia* - see Appendix A), belong to the fluorescent group. The fluorescent pseudomonads are characterised by their ability to produce a water soluble yellow-green fluorescent pigment and were first described by Flugge in 1886, who was

able to distinguish between two biotypes based on their ability to liquefy gelatin. These two biotypes now bear the names *P. putida* (non-liquefying) and *P. fluorescens* (liquefying) (Stanier *et al* 1966).

As demonstrated by den Dooren de Jong in 1926, the simple fluorescent pseudomonads are nutritionally versatile and are capable of utilizing a wide range of simple organic compounds as sole carbon sources (Palleroni 1978). Over 100 such sources have been tested and found to be metabolized by at least one pseudomonad (Stanier *et al* 1966). As do most Gram-negative bacteria, pseudomonads possess the ability to undergo changes in their colony morphology. The transformation of pseudomonads from a 'smooth' colonial form to a 'rough' colonial form is well documented and may be due to an alteration in the composition of the cell lipopolysaccharide wall layers (Jarrell and Kropinski 1977), although the mechanism by which this occurs is unknown (Govan *et al* 1979). In the case of the mushroom pathogens *P. tolaasii* and *P. gingeri*, the transformation from rough to smooth is associated with many physiological changes, among them a loss of virulence due to a cessation of toxin production (Cutri *et al* 1984). Surprisingly the rough avirulent form of *P. tolaasii* has been shown to promote *Agaricus* mycelial growth in a similar manner to the saprophytic *P. putida* (Rainey 1989). Stolp (1961) (in Lelliott *et al* 1966), in considering the fluorescent pseudomonads as a homogeneous group, believed that the pathogenic forms may arise from the saprophytes via mutation. Regarding this view as rather simplistic, Lelliott *et al* (1966), however, suggested the derivation of smooth virulent bacteria from the rough saprophytes may be driven by such selective pressures as mushroom exudates. The rough forms appear to be more tolerant to such environmental pressures as nutrient stress, a situation common in the nutrient poor and highly competitive mushroom casing (Rainey 1989).

Another fluorescent pseudomonad of note is *P. agarici*, the causal organism of 'drippy gill' disease of the cultivated mushroom *Agaricus* spp. Symptoms expressed possess several unusual features which sets this disease apart from the commom blotches. The present study is aimed at clarifying certain aspects of the aetiology and epidemiology of this disease.

CHAPTER TWO

'DRIPPY GILL' SYMPTOMOLOGY AND THE BIOLOGY OF THE CAUSAL ORGANISM

2.1 INTRODUCTION

2.1.1 'Drippy Gill' Symptomology

Young (1970) described the development of 'drippy gill' disease on *Agaricus bisporus* sporocarps. In the early stages of infection, small dark brown or black round spots appear on the sides and bottom edges of the lamellae of mushroom hymenia and develop to a diameter of 2mm or more. At the centre of each spot, a grey droplet composed of bacteria develops which may span the space between two adjacent lamellae. This droplet is the characteristic feature of the disease and from it is derived the common name 'drippy gill'. In severe infections, individual droplets coalesce and form ribbons of slime, followed by the breakdown of the gill tissue.

Infection, in some cases, tends to inhibit cap development and distort the pileus, while in other occurrences, depending chiefly upon the time of infection, damage appears minimal but gill tissue is of a pale colour, indicating the failure of the hymenium to mature. Other than cap distortion, damage to the pileal tissue is confined entirely to the gills.

The stipes of infected mushrooms are characterized by longitudinal splits which may reach the order of 2cm in length. As the mushroom matures, these splits become dark brown and the shiny inner surfaces are coated with the causative organism.

P. agarici is not confined solely to *Agaricus* as host. Bessette *et al* (1985) reported the occurrence of yellow blotch disease of the oyster mushroom *Pleurotus ostreatus* from a commercial farm in California, caused by *P. agarici*. The symptoms of this disease differ to those of 'drippy gill' in that the orientation of the sporocarp is affected and a clear yellow fluid is produced on the surface of the sporocarp cluster and not solely on the hymenial surface. The stipe diameter and length are often reduced and become brittle and/or fibrous.

2.1.2 Recorded Outbreaks of 'Drippy Gill' Disease

In 1958, two mushroom farms in Kent, England, reported a disease characterized by oozing and necrotic hymenial tissue. The disease recurred on one of the two farms in 1963 and occurred on different farms in 1963, 1966 and 1967 (Bateson *et al* 1972). Bacteria isolated from affected gills consistently elicited identical symptoms when reinoculated into excised sporocarps. One isolate, however, was reportedly capable of blotching the mushroom cap in addition to producing 'drippy gill' symptoms. A further outbreak of brown blotch was described by these workers from Berkshire in 1971, from which was isolated a pseudomonad with characteristics similar to the 'drippy gill' organism.

In New Zealand, diseased mushrooms bearing similar symptoms, with the exception of blotching, were received by Plant Diseases Division, Auckland, from a commercial grower in June of 1968. In July and August of the following year, serious outbreaks were reported from the Christchurch, Wairarapa and Auckland areas (Young 1970).

O'Riordain (1972a; 1972b) recorded the occurrence of 'drippy gill' on mushroom farms in Ireland, but noted the outbreak did not seem to cause severe losses in comparison to the earlier reported incidence of the disease in New Zealand.

In November of 1991, sporocarps bearing 'drippy gill' symptoms were reported from a commercial grower in Christchurch, New Zealand. Symptoms expressed were identical to those described by Young (1970). Again, no major losses were incurred.

2.1.3 Description and Taxonomic Status of *Pseudomonas agarici* Young - The 'Drippy Gill' Causal Bacterium

The 'drippy gill' causal organism was isolated and described by Young (1970) as a *Pseudomonas* sp. based upon polar flagellation and the production of a diffusable fluorescent pigment. Results of subsequent tests examining nutritional, biochemical and pathogenic characteristics differed from those previously published for other fluorescent pseudomonads sufficiently for Young to assign the causative bacterium to a proposed new species, *P. agarici*.

Bateson *et al* (1972) classified their blotching isolate from the Berkshire outbreak in 1971 as possessing characters typical of group III of the LOPAT determinative scheme of Lelliott *et al* (1966). Whether the blotch symptoms they described were expressed in conjunction with characteristic 'drippy gill' symptoms is unclear. The connection is implied in that the causal organism was found to belong to LOPAT group III to which *P. agarici* is also ascribed. Bateson *et al* (1972) also noted a 'close similarity' between the 'drippy gill' causal organism and *P. cichorii*, an established group III member.

Modern bacterial taxonomic methods based on rRNA analyses *show P. agarici* is closely related to representatives of rRNA branch I (De Vos *et al* 1985). *P. agarici* is relegated to Section V in Bergey's Manual of Systematic Bacteriology (2nd edition 1984), which it shares with, amongst others, *P. tolaasii*, the causal organism of mushroom brown blotch. Section V appears to be the 'dumping ground' for the pseudomonads of "....whose natural relationships with well characterized species of this genus are largely unknown", and consequently, the classification of members of this group is based on source of isolation (Palleroni 1984).

2.1.4 PV29: A Bacterium Causing Symptoms Similar to *P. agarici*

In 1988, an outbreak of an unusual bacterial disease of cultivated mushrooms was reported from the North Island of New Zealand (Rainey and Cole 1988). Though the tissues of PV29 affected sporocarps were identical to those affected by 'drippy gill', the degree of expression of symptoms in the affected tissues was dissimilar. PV29 was reported as causing malformations of the sporocarp and disruption and constriction of the hymenium, but without the prevalence of the profuse bacterial ooze characteristically associated with 'drippy gill' affected hymenia. Similarly, PV29 reportedly affected the stipe in a different manner to *P. agarici*. PV29 affected stipes showed greater tissue degradation seemingly without the distinctive longitudinal splitting, so evident in 'drippy gill', though both conditions generate copious bacterial ooze from the stipes.
Attempts to elucidate the taxonomic position of this novel isolate led Rainey and Cole (1988) to propose the formation of a new subdivision of LOPAT group III (to which *P. agarici* belongs) to accommodate PV29.

2.1.5 Evidence Supporting the Intrahyphal Occurrence of *P. agarici*

The nature of the symptoms caused by *P. agarici* on *Agaricus* spp. suggests the causal organism may occur and indeed be transmitted intrahyphally. Symptoms are often expressed before veil break (Young 1970), hence before exposure to external pathogens which implies the bacteria are already present within the mycelium at this stage. Furthermore, 'drippy gill' symptoms are confined only to the hymenium and stipe of affected sporocarps unlike proven extrahyphal bacterial pathogens such as *P. tolaasii*. Watering of sporocarps infected with *P. tolaasii* soon spreads blotch symptoms over the entirety of the cap.

Watering does not seem to affect the degree of coverage in the case of *P. agarici*.

Agaricus sporocarp development is considered to be bivelangiocarpic (Watling 1984). At early stages of development, the embryonic tissues are protected by two membranes, one of which specifically envelopes the maturing hymenial tissues. It is unlikely, therefore, that the stipe and hymenium are exposed to external pathogens during early development.

If *P. agarici* was found to be intrahyphal, this would not be a novel observation as the pathogen causing 'mummy' disease, another fluorescent pseudomonad, has been demonstrated, among others, to occur intracellularly (section 3.1.5).

2.1.6 The Dolipore-Parenthesome Septum: A Barrier to Intrahyphal Transmission?

Agaricus mycelium demonstrates a complex septation unique to Basidiomycetes, which is found in many representatives of this group. The septum consists of a dolipore delineated by a pair of opposing swollen barrel-shaped wall protuberances which are encapsulated on either side of the septum by a parabolic-shaped perforate membrane, the parenthesome. The parenthesome, in part, is continuous with the endoplasmic reticulum complement of the fungal cell. This component of the complex septum demonstrates the greatest structural variation (Moore 1984), particularly with reference to the degree of perforation exhibited. Within the dolipore itself are often electron-dense occlusions or pore plugs described as being composed of a proteinaceous material and their presence is recognized as being tissue type dependent (Flegler *et al* 1976) as opposed to a function of age or stress (Butler and Bracker 1970).

Dolipore-parenthesome septa are thought to play a regulatory role in nuclear migration ensuring the maintainence of a stable nuclear condition yet also allowing cytoplasmic continuity between adjacent hyphal cells

(Bracker 1967; Mayfield 1974). Pore occlusions may act as a regulatory mechanism preventing, or at least limiting protoplasmic streaming in vegetative mycelium and allowing increased streaming when fruiting is initiated (Flegler *et al* 1976). Being of a proteinaceous nature, pore occlusions are easily digested when necessary and thus perform as an 'on switch', controlling cytoplasmic streaming.

Passage of bacteria through the dolipore-parenthesome septum is restricted not only by the diameter of the perforations in the parenthesome and by the diameter of the dolipore itself, but also by the pore occlusions when present. Measurements of dolipore-parenthesome septal dimensions of *Agaricus bisporus* (Patton and Marchant 1978) show the mean septal pore diameter to be 113nm while the parenthesome holes measure, on average, 80.1nm in diameter, inadequate to accommodate a bacterial cell measuring some 0.5μm x 2.0μm. Evidence that dolipore-parenthesome septa are broken down by the parent fungus naturally has been presented by Giesy and Day (1965). They found septa in heterokaryotic mycelium of *Coprinus lagopus* exhibiting a range of complexity from simple septa to complete dolipore-parenthesome septa. They surmized that the intermediate structures observed represented intermediate stages in the breakdown of complex dolipore-parenthesome septa to simple septa which facilitates the rapid migration of intact nuclei.

This chapter investigates the basic biology and pathology of the 'drippy gill' causal organism, and its occurrence within *Agaricus* sporocarp tissue. The relationship between *P. agarici* and PV29 is studied and conclusions drawn about their taxonomic status.

2.2 MATERIALS AND METHODS

2.2.1 'Drippy Gill' Symptomology

Mushroom sporocarps, at the open cup stage of development, were purchased from a commercial mushroom grower in Christchurch, New Zealand, in November 1991. Initially, they appeared to be healthy, clean mushrooms. While being prepared for cooking it was noticed that the gills were exuding a bacterial slime while splits had appeared in the stipes (Alan Dickson, Department of Plant and Microbial Sciences, University of Canterbury, pers. comm.). Upon receipt, infected sporocarps were closely examined both externally and internally. For microscopic investigation, sporocarps were sectioned and blocks of tissue approximately $2mm^3$ were removed from five regions of the sporocarp (Fig. 2.1) and prepared for light and electron microscopy.

Tissue Preparation:

1. Tissue blocks were fixed in 2.5% glutaraldehyde in 0.075M Sorensen's phosphate buffer (pH7.3) (Gomori 1955) for 2h at room temperature.
2. Following a buffer wash (3 changes of 0.075M PO_4, 10min each change), the tissue was post-fixed in 1% osmium tetroxide in 0.075M PO_4 buffer for 3h at room temperature, followed by a short buffer wash.
3. Tissue blocks were dehydrated through an ascending acetone series from 20% to 80% in 20% increments for 10min each, concluding with 3 changes of 100% acetone for 10min each.
4. Samples were then infiltrated with Spurr's resin (Spurr 1969) by subjecting the tissue blocks to 30% resin overnight on a rotator followed by 75% resin for several hours.
5. Embedding was carried out by immersing tissue blocks in 100% resin in shallow, flat vessels and polymerizing at 70°C for 24h.
6. After hardening, the tissue samples were removed from the resin block and glued onto resin stubs. Following trimming with a razor blade by hand, 3-4μm sections were cut (LKB Bromma 11800 Pyramitome) for light

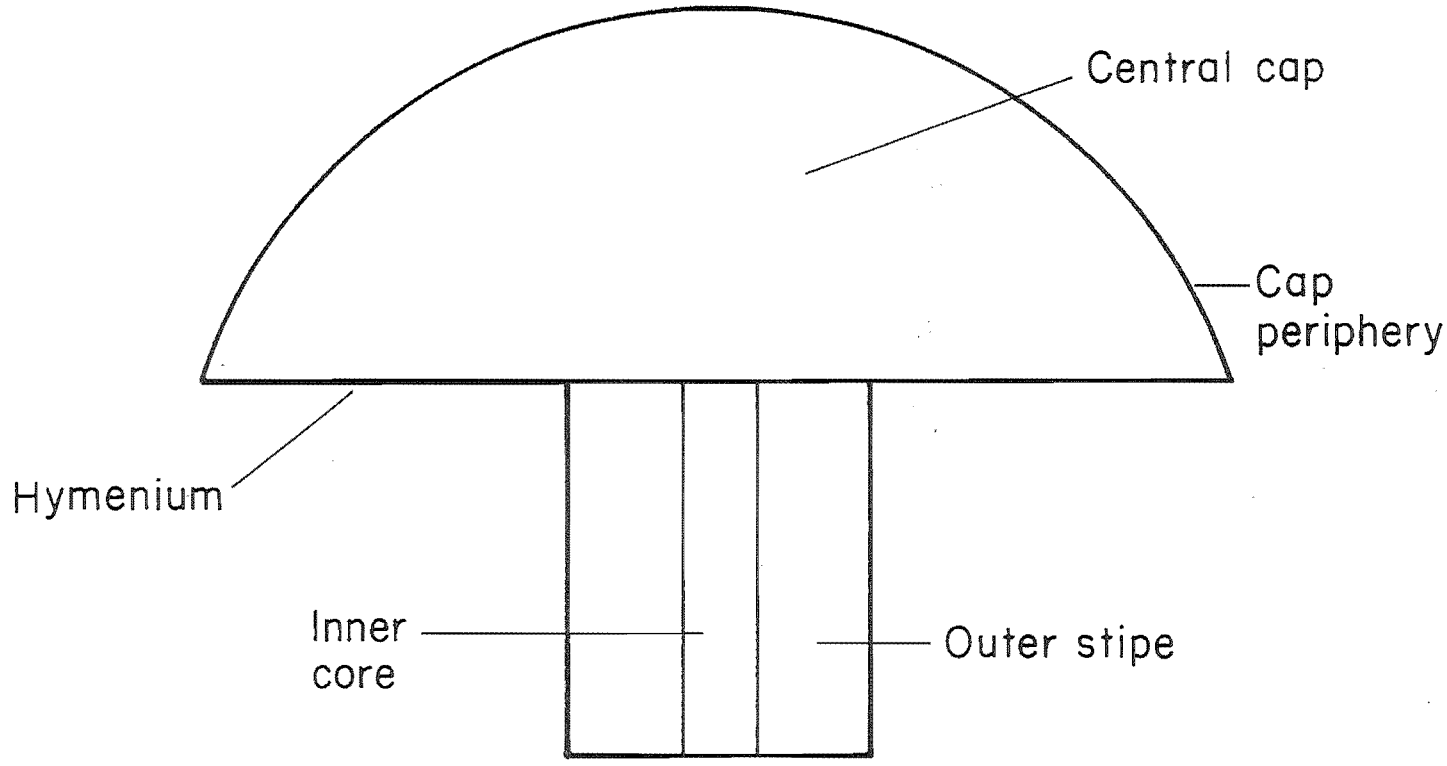


Figure 2.1 Location of tissues sampled from a 'drippy gill' affected sporocarp.

microscopy and stained with 1:1 methylene blue:azure blue for 2min and mounted in DPX.

7. To enhance resolution of the bacteria, light sections were examined and subsequent photomicrographs taken utilizing phase contrast optics attached to an Olympus BH2 microscope.

8. For TEM sections, mesas were cut by hand with a razor blade and thin sections (0.1μm on a LKB Bromma 2128 Ultrotome) floated onto Formvar coated 300 mesh copper grids. The sections were stained with 1% uranyl acetate in 100% ethanol (10min) followed by lead citrate (Sato 1967) for 5min. Grids were then dried and examined at 80kV in a Jeol JEM-1200EX electron microscope.

2.2.2 Isolation and Maintenance of the 'Drippy Gill' Pathogen

To isolate the causal bacterium, individual, discrete droplets from the hymenium were picked up on a sterile loop and streaked directly onto KB medium (King *et al* 1954). The plates were incubated in darkness at 25^{o}C for 48h and checked for purity. Individual colonies were then subcultured onto KB and incubated as above. After 48h, a single colony was transferred to 5ml NB in a universal tube and incubated in darkness in an orbital shaker (30^{o}C, 200rpm) for 24h. A 0.8ml aliquot of the subsequent suspension was transferred aseptically to an Eppendorf tube containing 0.2ml sterile glycerol and stored, in duplicate, at -80^{o}C. The bacterium was deposited in the bacterial collection of the Department of Plant and Microbial Sciences, University of Canterbury and designated CANU-PMS752. For short-term storage, the bacterium was suspended in 1/50 KB broth and kept at room temperature in darkness.

Other 'drippy gill' pathogens were procured from PDDCC Auckland, New Zealand and included in parts of this study. The Type Strain ICMP2656 (NCPPB2289; ATCC25941) was stored at -80^{o}C as described above and designated PMS601. ICMP2663 was similarly stored and designated PMS603. The PV29 organism (Paul Rainey, Department of Plant and

Microbial Sciences, University of Canterbury, pers. comm.) was stored and designated PMS133 but for convenience, was referred to as PV29.

2.2.3 Comparison of the 'Drippy Gill' Pathogens

Rainey and Cole (1988) reported several differentiating characteristics between *P. agarici* and PV29, upon which they proposed a creation of a new subdivision of LOPAT group III. The differentiating tests of Rainey and Cole (1988) were repeated and a wider range of standard biochemical tests and carbohydrate and organic acid salt utilization tests were completed.

The following tests were carried out: Size, Gram reaction and general morphology as revealed by Gram's staining; KOH solubility (Suslow *et al* 1982); motility in an infusion of mushroom tissue (Young 1970); flagellation by Ryu's stain (Kodaka *et al* 1982) and negative staining with 2% phosphotungstic acid followed by examination by transmission electron microscopy; presence/absence of poly-ß-hydroxybutyrate inclusions, levan production, starch hydrolysis, gelatin liquefaction, mushroom blotching and 'Tween' 80 hydrolysis (Lelliott and Stead 1987); fluorescence on KA and KB media (King *et al* 1954); arginine dihydrolase (Thornley 1960); DNAse (BBL); catalase (Smibert and Krieg 1981); potato soft rot (Lelliott *et al* 1966); H_2S from cysteine (Dye 1968); oxidase (Kovacs 1956); tyrosine hydrolysis (King and Phillips 1985); tobacco hypersensitivity and phenylalanine deaminase (Klement *et al* 1990); NO_3 reduction, indole production, urease production, ß-galactosidase production (p-nitrophenyl-ß-D-galactopyranoside utilization), lysine and ornithine decarboxylases and tryptophan desaminase (API 20E strips); utilization of caprate, adipate, malate, citrate and phenylacetate (API 20NE strips); glycerol, erythritol, D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, adonitol, ß-methyl-xyloside, galactose, D-glucose, D-fructose, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl-D-mannoside, α-methyl-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose,

inulin, melezitose, D-raffinose, amidon, glycogen, xylitol, ß-gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2 keto-gluconate, 5 keto-gluconate (API 50CH strips using the medium of Goor *et al* 1986); utilization of acetate, benzoate, alginate, and tartrate (Dye 1968).

The susceptibilty of the isolates to eleven common antibiotics (ampicillin, chloramphenicol, erythromycin, kanamycin, nalidixic acid, penicillin G, polymyxin B, rifampicin, spectinomycin, streptomycin and tetracycline) was tested in an effort to discern any major discrepancies between *P. agarici* and PV29 in their behaviour to these compounds. Five concentrations (0, 50, 100, 250 and 500μg/ml) of each antibiotic were made in the appropriate solvents. A 6mm sterile assay disc was placed in each solution and plated onto nutrient agar (NA) plates, seeded with the bacteria. The plates were incubated at 25^{o}C in darkness for 72h and the zones of inhibition were measured. The results are presented as a minimum concentration at which inhibition was noted. Each concentration of antibiotic was treated in triplicate.

2.2.4 Proof of Pathogenicity

A series of inoculations and isolations was carried out in an effort to satisfy Koch's postulates:

Excised *Agaricus* sporocarps were inoculated by applying a loopful of a single, pure colony of each of PMS752, PMS601, PMS603 and PV29 on to the cap surface and gently emulsifying it in a loopful of sterile distilled water. The sporocarps were placed into glass vials containing absorbent cotton wool and sterile distilled water and were then incubated at room temperature in a darkened box (approximately 15-20^{o}C, 84%RH) and watered daily with an atomiser until wet for 4-6d after which 'drippy gill' symptoms began appearing.

Alternatively, pure colonies of PMS752, PMS601, PMS603 and PV29 were suspended in 5ml of sterile distilled water and diluted to achieve an A_{620} of 0.4, corresponding to a density of approximately 10^{8} cells/ml. Excised

sporocarps were inoculated with the bacteria by injecting 100μl of suspension into the subhymenial tissues of the pileus and incubating them in glass vials as above.
In both instances, the causal bacterium was re-isolated in pure culture from hymenial droplets. Inoculations were repeated using identical methods as above and sporocarps were again incubated as outlined. From subsequent bacterial droplets, pure colonies of the causal bacteria were again isolated.

In an effort to confirm the causative nature of the bacterial strains, rifampicin resistant mutants were acquired by selecting for spontaneous mutants on antibiotic gradient plates (Carlton and Brown 1981) containing a range of 0-50μg/ml of rifampicin in NA. Each bacterial strain was streaked on to a gradient plate and incubated for 48-72h at 25^{o}C in darkness. Single isolated colonies were selected from approximately halfway along the gradient then subcultured to NA containing 100μg/ml rifampicin and incubated as above. Finally, single isolated colonies were transferred to NA containing 500μg/ml rifampicin in NA and incubated as before. Subsequent colonies were then stored at room temperature on NA slopes containing 100μg/ml rifampicin.

Excised sporocarps were inoculated by applying a single bacterial colony onto the cap and emulsifying it in a loopful of sterile distilled water. The sporocarps were incubated as previously and checked regularly for symptoms. Once droplets had appeared on the hymenium, the stipes were removed from the sporocarps and the hymenia touched lightly to the surface of NA plates containing 500μg/ml rifampicin. The plates were then incubated at 25^{o}C for 72h and inspected for growth.

2.2.5 Toxin Assay

The ability of fluorescent pseudomonads to produce a range of metabolites active against other organisms is well known. They are able to produce toxins (Nair and Fahy 1973; Brodey *et al* 1991), antibiotics (Gurusiddaiah *et*

al 1986) and bacteriocins (Vidaver *et al* 1972). In order to test for a possible antifungal agent produced by the 'drippy gill' isolates against *Agaricus* mycelium, they were each plated against the fungus and areas of inhibition noted. Compost malt medium (CMM) agar (Rainey 1989a) plates were inoculated with *Agaricus* mycelium by transferring a CMM agar plug from a stock plate and incubating it in darkness at 25^{o}C for 10d. Each 'drippy gill' isolate was then streaked onto the agar towards the outer periphery of the plate. Cell-free culture filtrates were prepared of each isolate by inoculating 5ml of nutrient broth (NB) with each bacterium and incubating them in an orbital shaker at 30^{o}C, 200rpm for 24h in darkness. They were then centrifuged (11,000g, 4^{o}C, 10min) and the resultant supernatant was filter sterilized through a 0.22μm Millipore membrane into sterile universal bottles. A 10mm sterile assay disc was introduced into each cell-free culture filtrate, touch dried and placed onto the agar against the side of the petri dish, opposite the streak. The plates were sealed with 'gladwrap' and incubated for a further 14d.

Cell-free culture filtrates were prepared as above and applied to blocks of *Agaricus* sporocarp tissue to observe any possible reaction of the 'drippy gill' metabolites against tertiary *Agaricus* mycelium. 20μl of each of the filtrates were pipetted onto approximately 5mm^3 pileal tissue blocks which were subsequently incubated in a humid chamber at 25^{o}C in darkness for 72h. Following this period, sections of inoculated tissue from each bacterial treatment were removed and prepared for light microscopy (2.2.1).

2.2.6 Microscopic Examination of 'Drippy Gill' Bacteria and *Agaricus* Mycelium

Two methods were employed to investigate the fungal bacterial interaction with light microscopy.

Firstly, for each 'drippy gill' isolate, a CMM agar plate was centrally inoculated with *Agaricus* mycelium and the bacteria streaked in a circle around the point of inoculum. The plate was sealed and incubated at 25^{o}C

in darkness until the mycelium had penetrated the ring of bacteria and colonized the agar outside the bacterial inoculum. Mycelium was then scraped from the agar surface outside the bacterial streak and vortexed in 1.0ml sterile distilled water in a sterile Eppendorf tube to dislodge any loosely adherent bacteria. The mycelium was then transferred to a drop of lactophenol cotton blue on a glass slide, a cover slip added and examined. Secondly, glass slides coated with agar were prepared. Clean, grease free slides were rested on glass elbows in glass petri dishes containing moistened filter paper and sterilized (121^{o}C, 15min). While the slides were still hot from autoclaving, molten CMM agar (approximately 80-85^{o}C) was poured into a sterile petri dish and each slide immersed into it. The slides were left to dry leaning on a sterile petri dish lid. Once the agar had set, the slides were inoculated at the centre with a 3mm plug of *Agaricus* mycelium. Overnight liquid cultures of the 'Drippy gill' bacteria were inoculated onto each slide by pipetting 10μl of suspension onto one edge of the slide and drawing it across with the pipette tip approximately 5mm on either side of the fungal inoculum, being careful not to break the agar film. The slides were incubated in the glass petri dishes at 25^{o}C in darkness until the mycelium had progressed through the bacterial culture. A drop of water was placed on each end of the slide and coverslips added. The material was examined using Nomarski differential interference optics attached to an Olympus BH2 microscope.

2.2.7 Growth Room Trials

Attempts were made to isolate the possible source of 'drippy gill' infection from the mushroom growing bed. Spawned compost (strain Lyon X20) and casing was acquired from a commercial grower and incubated in a purpose-built growing shed following recommended procedures (Paul Rainey pers. comm.). Four treatments were applied to the growth trays in triplicate.

A] The compost was inoculated before casing with 500ml of an overnight culture of *P. agarici* in NB at the rate of approximately 100ml/300cm^{2}

compost surface area. The trays were watered with clean water and covered with damp newspaper to ensure the compost did not dry out.

B] Following casing, the surface was inoculated as above and treated identically.

C] The mushroom trays were watered with a suspension of *P. agarici* (approximately 10^4 cfu/ml) in distilled water, which was repeated when watering was required.

D] Control trays - these were grown under normal conditions and kept separate from the test trays.

Resultant mushrooms were investigated at different developmental stages for 'drippy gill' symptoms.

2.3 RESULTS

2.3.1 'Drippy Gill' Symptomology

Examination of infected sporocarps showed a profuse bacterial ooze emanating from many of the hymenial lamellae. Discrete droplets of cream-coloured bacterial ooze in some cases coalesced with droplets from adjacent lamellae to form ribbons of 'slime' (Fig. 2.2a; 2.2d). Areas of some lamellae showed a darkening of the tissue precusory to droplet formation, while others showed similarly darkened areas to contain an embryonic droplet at or near their centre (Fig. 2.2a). Areas of lamellae in advanced stages of necrosis were noted, with either a light brown crusty appearance (Fig. 2.2a), or a moist black mucoid character (Fig. 2.2d) In both conditions, adjacent lamellae had coalesced at the point of necrosis. The different appearances of the tissue is suggestive of a secondary infection. Longitudinal splits evident in the stipes were often lined with a bacterial ooze identical in character to that of the hymenium (Fig. 2.2b). In severe cases, the splits ran the length of the stipe and pervaded the stipe to such an extent as to expose the inner core tissue (Fig. 2.2c)

Light Microscopy

Examination of internal tissues reveal copious bacteria, the majority of which were found to exist extrahyphally.

Tissues from the central cap, beneath the site of inoculation, show a heavy concentration of bacteria in the intercellular spaces (Fig. 2.3a). A number of scattered bacterial cells are visible within hyphae. The control section (Fig. 2.3b) gives some indication as to the area of extracellular matrix available to the bacteria, compared with the area of fungal cell. Isolated hyphae within the 'stream' of bacteria suggest the bacteria are unable to degrade the fungal wall material.

Tissue from the cap periphery (Fig. 2.4a), directly above hymenial droplets, shows a similar situation exists as to that found in the central cap. The majority of bacteria are confined to the intercellular spaces, which are again large and numerous (Fig. 2.4b), but in this case there is no evidence of intrahyphal existence.

The greatest damage is seen in the hymenium (Fig. 2.5a). Healthy basidia appear turgid and highly vacuolate (Fig. 2.5b), with a well organized subhymenium and tramal layer, the latter tissue possessing large intercellular spaces relative to the dimensions of the hyphae. 'Drippy gill' bacteria disrupt the organization of this tissue, making the trama and hymenium indistinct. Turgid, vacuolate basidia are absent from both sides of the lamella and hyphae within the bacterial mass have little recognisable structure. There are no intrahyphal bacteria evident in the hymenium. However, due to the disorganization of the tissue, it is difficult to distinguish discrete single hyphae.

The greatest aggregations of bacteria are found in the outer stipe tissue (Fig. 2.6a). The hyphae of the outer stipe are loosely packed, hence intercellular spaces are very large (Fig. 2.6b). Within the 'stream' of bacteria (Fig. 2.6a), can be seen isolated hyphae, which implies they are unaffected by the bacteria. However, particularly around the periphery of the 'stream' of bacteria, many hyphae are seen to have collapsed. The cause of this collapse is unclear, but it may be a result of pressure due to an increasing 'flow' of bacteria. Whether or not these hyphae have been degraded is impossible to ascertain, but there are signs of bacteria occurring intrahyphally. In the control section (Fig. 2.6b) collapsed hyphae similar to those observed in the 'drippy gill' outer stipe can be seen. Also visible is a discontinuity of the hyphal wall, a possible entry point for bacteria.

In longitudinal section (Fig. 2.7a), bacteria are again seen to be prolific, however, determining whether they are intrahyphal or not is very difficult. Bacteria overlying hyphae in a different plane of focus appear to be intrahyphal. Defining the exact outline of hyphae is also very difficult as they ramify through different planes of focus. The transparent nature of many of the hyphal cells makes it difficult to determine cytoplasm from

extracellular matrix. Outer stipe hyphae are typically highly vacuolate (Fig. 2.7b). The cells seen in Fig. 2.7a are not vacuolate, which may indicate these cells are unhealthy or damaged and perhaps susceptible to penetration by 'drippy gill' bacteria.

The tissues of the inner stipe remain remarkably free of bacteria (Fig. 2.8a). The close proximity of this tissue to the vast reservoir of bacteria found in the outer stipe suggests they are equally exposed to bacterial invasion. Like their near neighbours, inner stipe cells are typically highly vacuolate and loosely packed, leaving large intercellular spaces (Fig. 2.8b). The few bacteria seen in Fig. 2.8a are from the periphery of the inner stipe, virtually at the outer stipe-inner stipe interface. A pocket of bacteria are observed seemingly gaining entry to a damaged hyphal cell, but whether the damage is bacterially mediated, or a result of mechanical breakage is impossible to discern.

Tissues examined from the cap periphery which were not located over a hymenial droplet failed to show evidence of any bacteria. Similarly, central cap tissue, remote from the point of inoculation, also failed to show any presence of bacteria. Tissue from the central cap shows an interesting interaction between 'drippy gill' bacteria and *Agaricus* hyphal cells. The bacteria appear to be attached to the hyphal wall 'end on' (Fig. 2.9a) around the periphery of a large intercellular space, though adhesion was impossible to detect. Also evident are intrahyphal bacteria and a possible point of entry.

The largest intercellular spaces are found in the outer stipe tissue (Fig. 2.9b). It is this tissue which develops the longitudinal splits, having exposed faces coated with bacterial ooze. Stipe splitting may arise from these intercellular spaces following the colonization of the extracellular matrix by 'drippy gill' bacteria. A loss of integrity of the extracellular matrix may reduce the adhesion between hyphae and result in hyphae parting and the stipes eventually splitting. Damaged hyphae, which may allow entry to 'drippy gill' bacteria, are visible throughout the outer stipe.

Transmission Electron Microscopy

Transmission electron microscopy reveals 'drippy gill' bacteria to be prevalent in many tissues.

Within the stipe, 'drippy gill' bacteria are seen predominantly in the hyphal intercellular space (Fig. 2.10a), surrounding healthy, vaculoate fungal cells. Individual bacterial cells may be intrahyphal (Fig. 2.10b). Even when in close proximity to fungal walls, there is often no detrimental effect to the fungal cell (Fig. 2.10c). In other examples, however, the bacteria are seen to elicit a swelling from the fungal cell (Fig. 2.10d).

In the hymenium, the bacteria are again extrahyphal. In this case, however, there appears to be evidence of hyphal wall penetration (Fig. 2.11a). A closer investigation reveals the break to be a collapsed and folded hyphal wall (Fig. 2.11b).

Within the pileus, the bacteria again appear extrahyphal. There is evidence of the degradation of the extracellular matrix in the immediate vicinity of the bacteria (Fig. 2.11c). Despite the close proximity of the bacteria to fungal walls, they seem to have no detrimental effects on the fungal cellular organization. Distinguishing extrahyphal bacteria from intrahyphal bacteria is difficult in some cases (Fig. 2.11d), as an intercellular space is very similar in size and shape to a fungal hypha. Close examination of the fungal wall is required to clarify the situation.

There is no obvious adhesion of 'drippy gill' bacteria to the *Agaricus* cell wall, though there is some evidence of an interaction between the two organisms (Fig. 2.12a). It is evident that 'drippy gill' bacteria do occur intrahyphally and their entry can be demonstrated (Fig. 2.12b). While this example demonstrates entry probably via a pre-existing wall break, the bacteria have been demonstrated to enter possibly by actively penetrating the hyphal wall (Fig. 2.13). The bacteria within the fungal hypha are surrounded by an amorphous electron dense material which is presumably host cytoplasm, containing small, coiled structures. The outer bacterial membranes are clearly visible surrounding each individual cell and a flagellar remnant can be identified. The bacterium which appears to be

entering is deforming to penetrate the breach and displacing cytoplasmic material as it does so. Microfibrils, presumably of hyphal wall origin, are released to the extracellular space. A membranous structure, perhaps the fungal plasma membrane, is also being displaced. The bacterial cell itself, however, does not possess the crenulate outer envelope characteristic of the 'drippy gill' bacteria (Fig. 2.11b).

2.3.2 Description and Comparison of *P. agarici* and PV29

All three *P. agarici* isolates and PV29 are Gram negative and KOH positive small rods (0.5-1.0 x 1.25-1.75μm), motile by several polar flagella and do not accumulate poly-ß-hydroxybutyrate. They produce mucoid buff/straw coloured domed colonies and a weakly fluorescent diffusable pigment on KB (Figs. 2.14a, 2.14b). On KB, after several days, a small number of colonies transformed to give the characteristic sectoring into distinct smooth and rough colony morphologies. Attempts to maintain the rough variant as a pure culture were unsuccessful despite regular subculturing onto various media over a nine month period. Colonies isolated in this way took on a characteristic 'fried egg' appearance, suggesting a mixed culture of both smooth and rough variants.

The results of the biochemical and utilization tests are identical for all isolates (Table 2.1). Particular notice was taken of the tests which Rainey and Cole (1988) used to differentiate between *P. agarici* and PV29. Potato soft rot, NO_3 reduction and gelatin liquefaction yielded negative reactions for all isolates. Although the isolates hydrolyse tyrosine, the pigment produced was variable and dependent upon length of incubation. Also of interest was the tobacco hypersensitive reaction test, which Bateson *et al* (1972) believed initially to be positive for their 'drippy gill' isolates. As this test constitutes one of the LOPAT characteristics, allocation of the bacteria to the correct LOPAT grouping depends on this test. Following subculturing, their isolates lost the ability to elicit a hypersensitive response from tobacco. All isolates in this study yielded negative results for this test (Fig. 2.15) and confirmed all isolates as LOPAT - + - - - and therefore members of group III.

Table 2.1 Biochemical and Nutritional Comparison of *P. agarici* and PV29

	PMS 601	PMS 603	PMS 752	PV29
Fluorescence on KB	+	+	+	+
Fluorescence on KA	-	-	-	-
NO_3 Reduction	-	-	-	-
Tobacco Hypersensitivity	-	-	-	-
Tyrosine Hydrolysis	+	+	+	+
Starch Hydrolysis	-	-	-	-
`Tween' 80 Hydrolysis	-	-	-	-
Indole Production	-	-	-	-
H_2S (from cysteine) Production	+	+	+	+
Levan Production	-	-	-	-
Gelatin Liquefaction	-	-	-	-
Potato Softrot	-	-	-	-
Mushroom Tissue Blotching	-	-	-	-
Lysine Decarboxylase	-	-	-	-
Ornithine Decarboxylase	-	-	-	-
Phenylalanine Deaminase	-	-	-	-
Tryptophan Desaminase	-	-	-	-
β-Galactosidase	-	-	-	-
Catalase	+	+	+	+
DNAse	-	-	-	-
Urease	-	-	-	-
Oxidase	+	+	+	+
Arginine Dihydrolase	-	-	-	-
UTILIZATION OF:				
Acetate	+	+	+	+
N-Acetylglucosamine	-	-	-	-
Adipate	-	-	-	-
Adonitol	-	-	-	-
Alginate	-	-	-	-
Amidon	-	-	-	-
Amygdalin	-	-	-	-
D-Arabinose	-	-	-	-

Table 2.1 Continued

	PMS 603	PMS 601	PMS 752	PV29
L-Arabinose	-	-	-	-
D-Arabitol	+	+	+	+
L-Arabitol	-	-	-	-
Arbutin	-	-	-	-
Benzoate	+	+	+	+
Caprate	+	+	+	+
Cellobiose	-	-	-	-
Citrate	+	+	+	+
Dulcitol	-	-	-	-
Erythritol	-	-	-	-
Esculin	-	-	-	-
D-Fructose	-	-	-	-
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
Galactose	-	-	-	-
β-Gentibiose	-	-	-	-
Gluconate	+	+	+	+
2 keto-Gluconate	-	-	-	-
5 keto-Gluconate	-	-	-	-
D-Glucose	-	-	-	-
α-methyl-D-Glucoside	-	-	-	-
Glycerol	+	+	+	+
Glycogen	-	-	-	-
Inositol	-	-	-	-
Inulin	-	-	-	-
Lactose	-	-	-	-
D-Lyxose	-	-	-	-
Malate	+	+	+	+
Maltose	-	-	-	-
Mannitol	+	+	+	+
D-Mannose	-	-	-	-
α-methyl-D-Mannoside	-	-	-	-
Melezitose	-	-	-	-
Melibiose	-	-	-	-

Table 2.1 Continued

	PMS 603	PMS601	PMS 752	PV29
Phenylacetate	-	-	-	-
D-Raffinose	-	-	-	-
Rhamnose	-	-	-	-
Ribose	+	+	+	+
Saccharose	-	-	-	-
Salicin	-	-	-	-
Sorbitol	-	-	-	-
L-Sorbose	-	-	-	-
D-Tagatose	-	-	-	-
Tartrate	-	-	-	-
Trehalose	-	-	-	-
D-Turanose	-	-	-	-
Xylitol	-	-	-	-
D-Xylose	-	-	-	-
L-Xylose	-	-	-	-
β-methyl-Xyloside	-	-	-	-

Table 2.2 Antibiotic Sensitivity of *P. agarici* and PV29

	BACTERIAL ISOLATE			
ANTIBIOTIC[a]	PMS 601	PMS 603	PMS 752	PV29
Ampicillin	-	-	-	-
Chloramphenicol	-	-	-	-
Erythromycin	-	-	-	-
Kanamycin	50	50	50	50
Nalidixic Acid	-	500	-	-
Penicillin G	-	-	-	-
Polymyxin B	500	100	-	500
Rifampicin	250	250	250	250
Spectinomycin	-	-	-	-
Streptomycin	50	50	50	50
Tetracycline	50	250	250	100

[a] Minimum inhibitory concentration (μg/ml)

- Represents a minimum inhibitory concentration in excess of 500μg/ml

The antibiotic sensitivity assay demonstrated some variation in response by the bacteria (Table 2.2). Polymyxin B and tetracycline produced the greatest differences but these were insufficient to differentiate between *P. agarici* and PV29.

2.3.3 Proof of Pathogenicity

Koch's postulates, identifying *P. agarici* and PV29 as the causal organisms of 'drippy gill' disease of *Agaricus* sporocarps were completed. Both methods of artificial inoculation led to the 'drippy gill' syndrome. Artificial inoculations of excised sporocarps resulted in characteristic 'drippy gill' symptoms on the hymenia within 6 days incubation. Expression of symptoms on the stipe was minimal, severity ranging from no obvious symptom to the appearance of vertical striations similar in character to water-soaked tissue, possibly prefatory to longitudinal fissures. Hymenial droplets, transferred from infected sporocarps, were found to be largely composed of *P. agarici* bacteria. However, another fluorescent pseudomonad, *P. tolaasii*, a bacterium endemic to the sporocarp microflora, was also identified, which made up a very minor component of the bacterial ooze.

Inoculations of *Agaricus* sporocarps with rifampicin resistant *P. agarici* and PV29 isolates confirmed Koch's postulates. Bacteria constituting the hymenial droplets were shown to be rifampicin resistant and thus grew on rifampicin augmented NA (Fig. 2.16). The control plate, inoculated with the hymenium of an uninoculated sporocarp, did not show any evidence of bacterial colonization.

2.3.4 Toxin Assay

None of the four 'drippy gill' isolates show evidence of inhibition of *Agaricus* mycelium. However, PV29 showed definite signs of enhancing mycelial growth (Fig. 2.17a), indicated by the thick corded hyphae and profuse mycelium over the streak compared with the surrounded mycelium. The cell-free culture filtrate did not have a similar effect on the mycelium. The *P. agarici* filtrates gave little indication of promoting mycelial growth and were identical to PMS601 (Fig. 2.17b).

2.3.5 Microscopic Examination of 'Drippy Gill' Bacteria and *Agaricus* Mycelium

Lactophenol cotton blue preparations demonstrate the ability of the 'drippy gill' organisms (in this case PMS603) to penetrate *Agaricus* fungal hyphae (Figs. 2.18a, 2.18b). Prior to gaining entry to the hyphal cell, the bacteria are seen to 'attach' themselves to the outside of the mycelium 'end on' (Fig. 2.18a). Not only does this occur around occupied cells, but also the cells which remain clear of bacteria. The septum of the occupied cell remains intact. At no stage, however, were more than two adjacent hyphal cells seen to be occupied by bacteria. The septum between the two cells appears to be intact (Fig. 2.18b), though it is impossible to detect any degradation of the parenthesome or dolipore.

Interference microscopy reveals no intrahyphal bacteria, but does demonstrate the predilection 'drippy gill' bacteria seemingly have for *Agaricus* mycelium (Fig. 2.18c). They appear to surround the hyphal cell, but not positioned perpendicular to it as seen in the previous preparation.

The dolipore parenthesome septum would appear to present a physical barrier to movement of bacteria between cells (Fig. 2.19). The parenthesome pores of the septum in Fig. 2.19 average 65nm, the dolipore

at its widest measures approximately 0.18μm and the dolipore at its narrowest measures approximately 52nm. This would prevent passage of nuclei and bacteria.

2.3.6 Growth Room Trials

Attempts at identifying a possible source of inoculum of 'drippy gill' in simulated commercial growing conditions did not give a clear result. From inoculated compost, symptoms were observed sporadically within the third flush and from the inoculated casing symptoms were observed from the second and third flushes. The water-borne inoculum treatment yielded two sporocarps from the third flush with water-soaked lesions on the hymenium, resembling the early stages of droplet formation. The control resulted in three flushes of healthy, well-formed sporocarps. Sporocarps examined for symptoms prior to veil break showed no evidence of the disease.

FIGURE 2.2

FIG. 2.2a: Hymenium of *A. bisporus* sporocarp affected by *P. agarici.* Bacterial droplets (BD), forming on the lower edge of the lamellae, emanate from the centre of areas of darkened tissue (DT). Embryonic droplets (ED) are seen developing from these areas. Necrotic tissue (N) is visible where adjacent lamellae touch. Apparent putrefaction may be in part due to colonization by other bacteria.

FIG. 2.2b: Infected *Agaricus* sporocarp demonstrating the two major symptoms of 'drippy gill'. Bacterial droplets (BD) on the hymenium and a longitudinally split stipe issuing bacterial ooze (BO).

FIG. 2.1c: Symptom expression on the stipe of *Agaricus*. Symptoms range from longitudinal streaking to deep pervasive splits exposing the internal core stipe tissues (IC).

FIG. 2.2d: Bacterial droplets on *Agaricus* hymenium. Often they coalesce with those on adjacent lamellae and form ribbons of 'slime' (RS), which may eventually drip onto the mushroom bed. Areas of necrosis (N) are apparent often at points where adjacent lamellae touch.

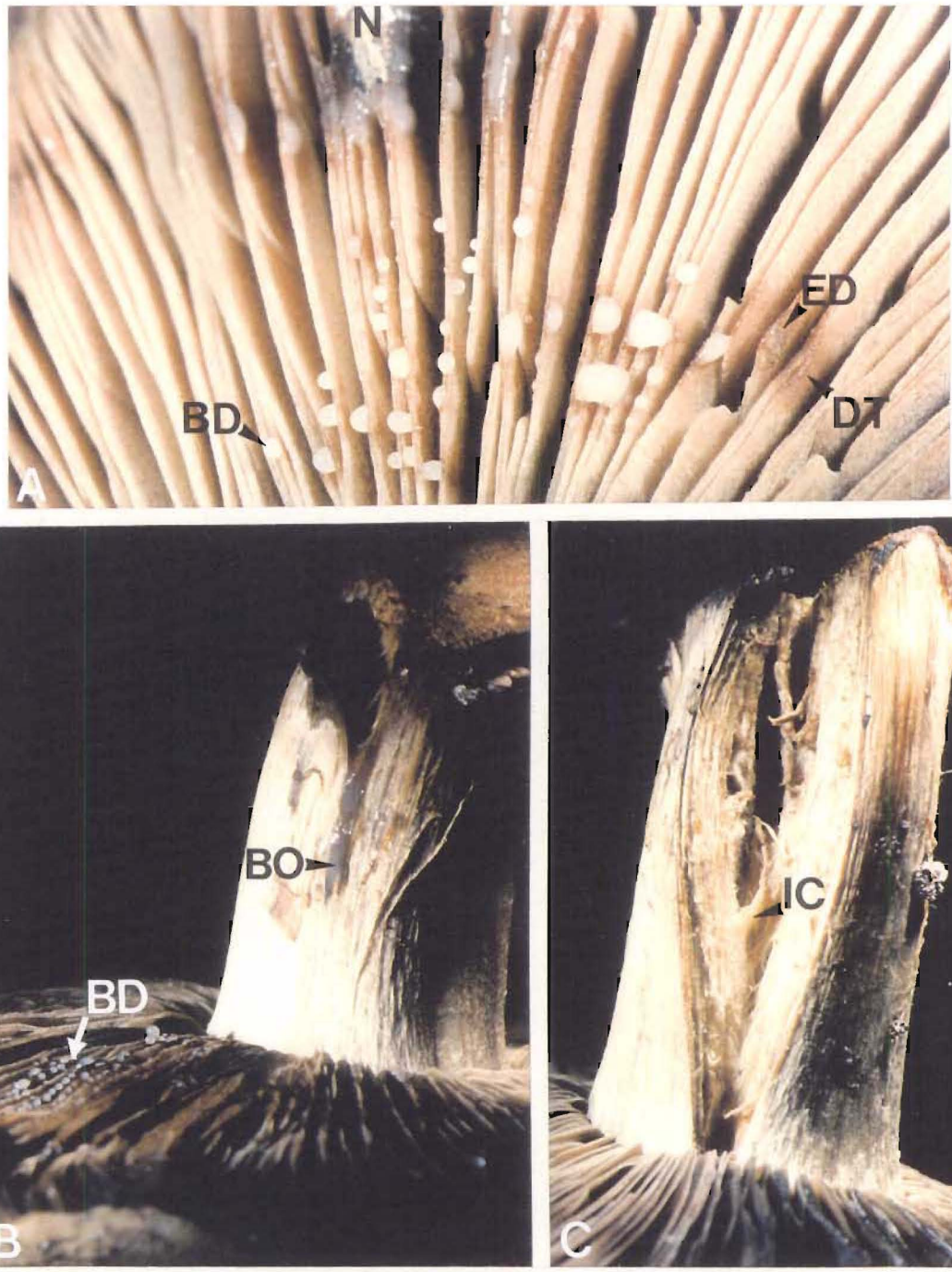
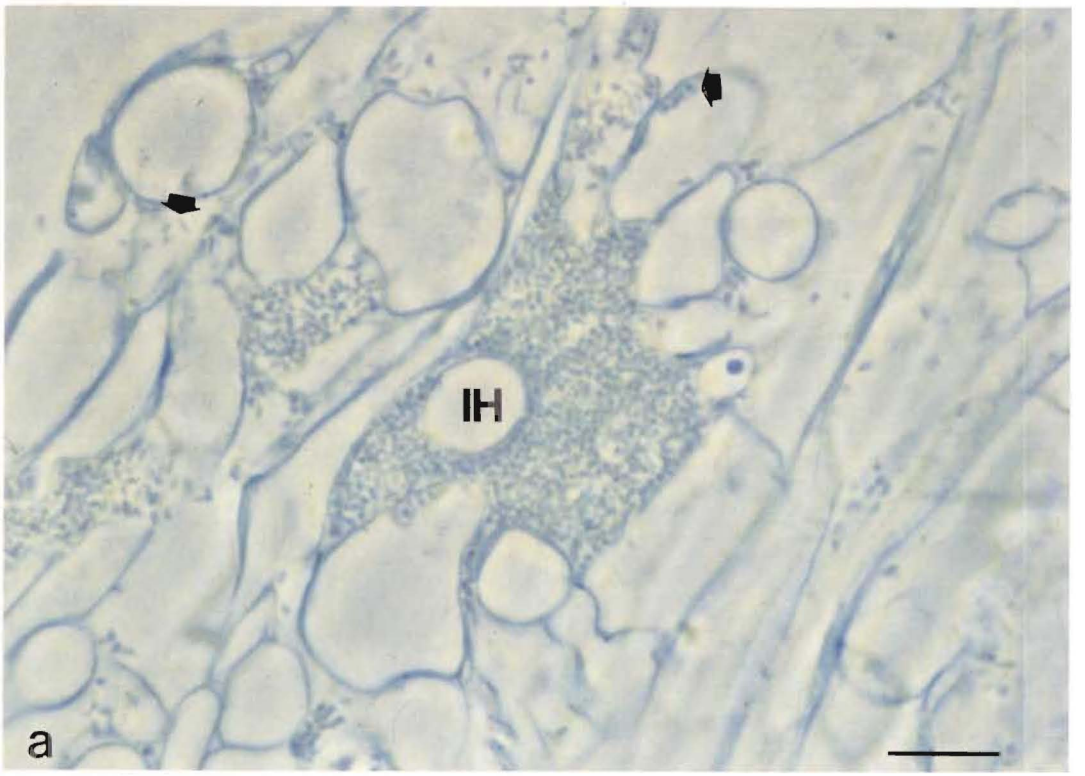

N
ED
DT
BD
A
BO
BD
B
IC
C
RS
N
D

FIGURE 2.3

Fig. 2.3a 'Drippy gill' bacteria in *Agaricus* central cap tissue. The majority of bacteria are extrahyphal, although some can be seen within hyphae (arrows). Note the isolated hyphae (IH) within the 'stream' of bacteria. Bar = 10μm.

Fig 2.3b *Agaricus* central cap hyphae from a 'clean' sporocarp. Note the large intercellular spaces (IS). Bar = 10μm.

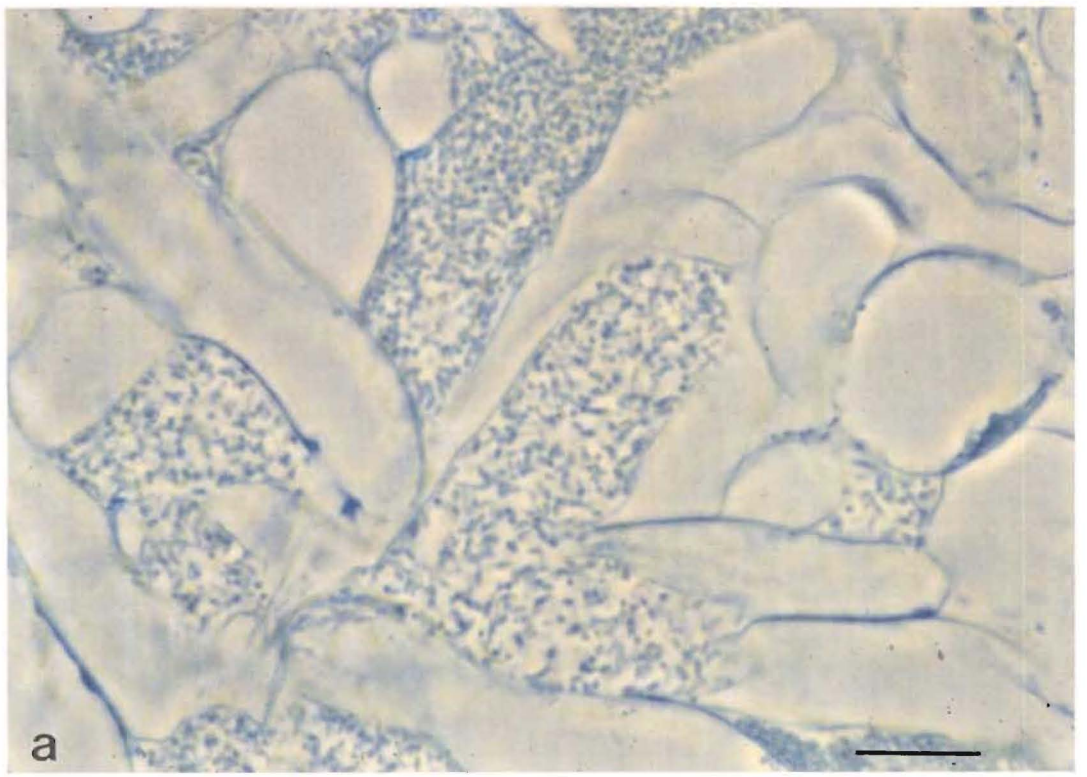

IH
a


IS
b

FIGURE 2.4

Fig. 2.4a 'Drippy gill' bacteria in *Agaricus* peripheral cap tissue. The bacteria are confined to the intercellular spaces, with no evidence of intrahyphal occurrence. Bar = 10μm.

Fig 2.4b *Agaricus* peripheral cap hyphae from a 'clean' sporocarp. Note the large intercellular spaces (IS). Bar = 10μm.


a

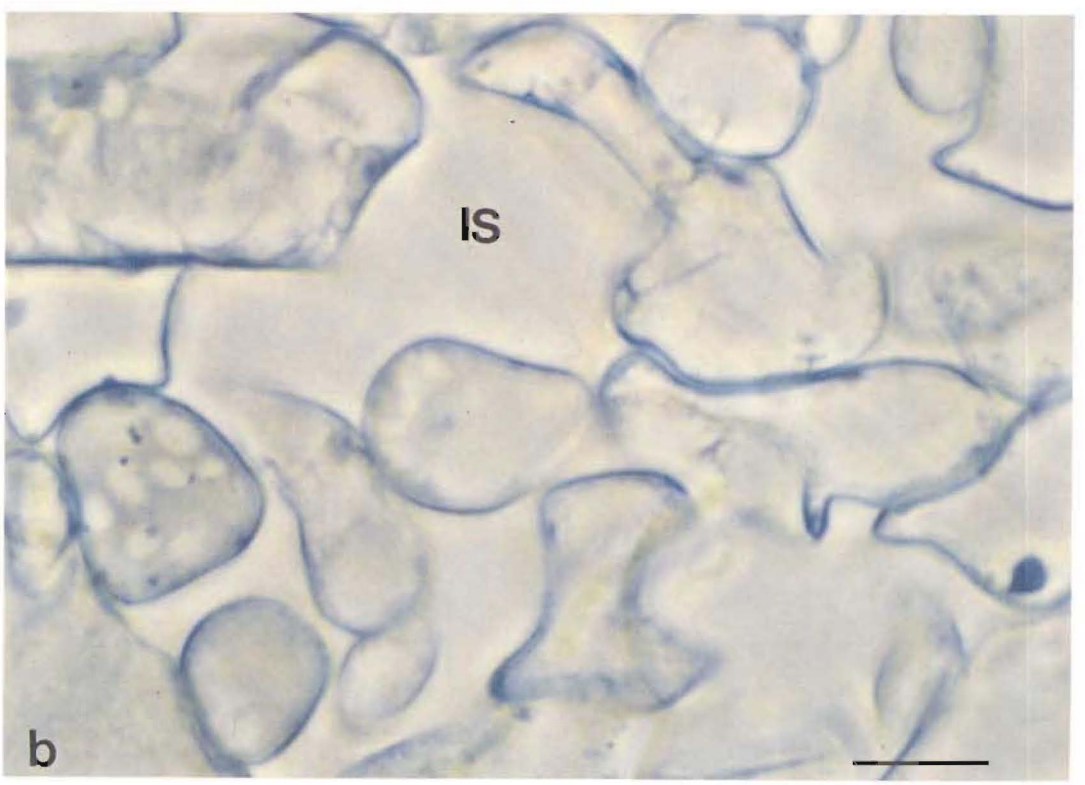
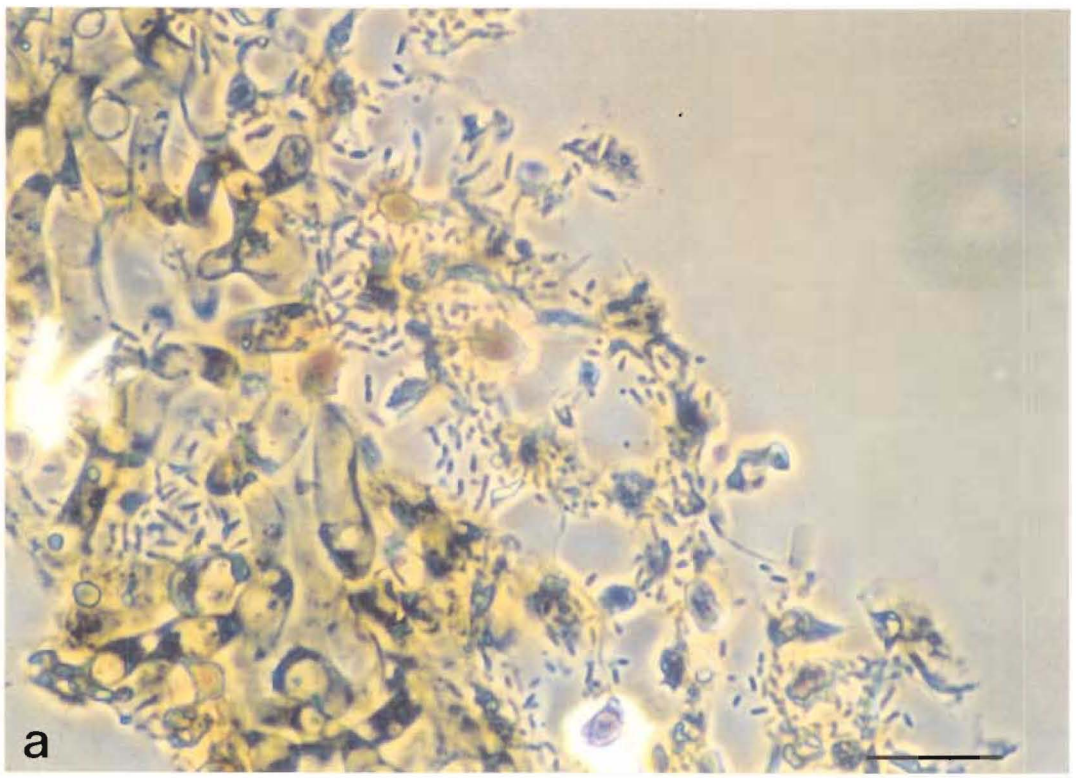

IS
b

FIGURE 2.5

Fig. 2.5a 'Drippy gill' bacteria in *Agaricus* hymenial tissue. The three tissue types of the hymenium are indistinguishable and individual hyphae are indistinct. Bar = 10μm.

Fig 2.5b *Agaricus* hymenial tissue from a 'clean' sporocarp. The different tissue layers are discrete and distinguishable. The trama (T) is composed of anastomosing hyphae with relatively large intercellular spaces; the subhymenium (SH) is composed of hyphae with a greater diameter and are more closely packed than the trama; the hymenium (H) is characterised by the large, erect, turgid basidia (B) producing basidiospores (BS) on sterigmata. Bar = 10μm.


a

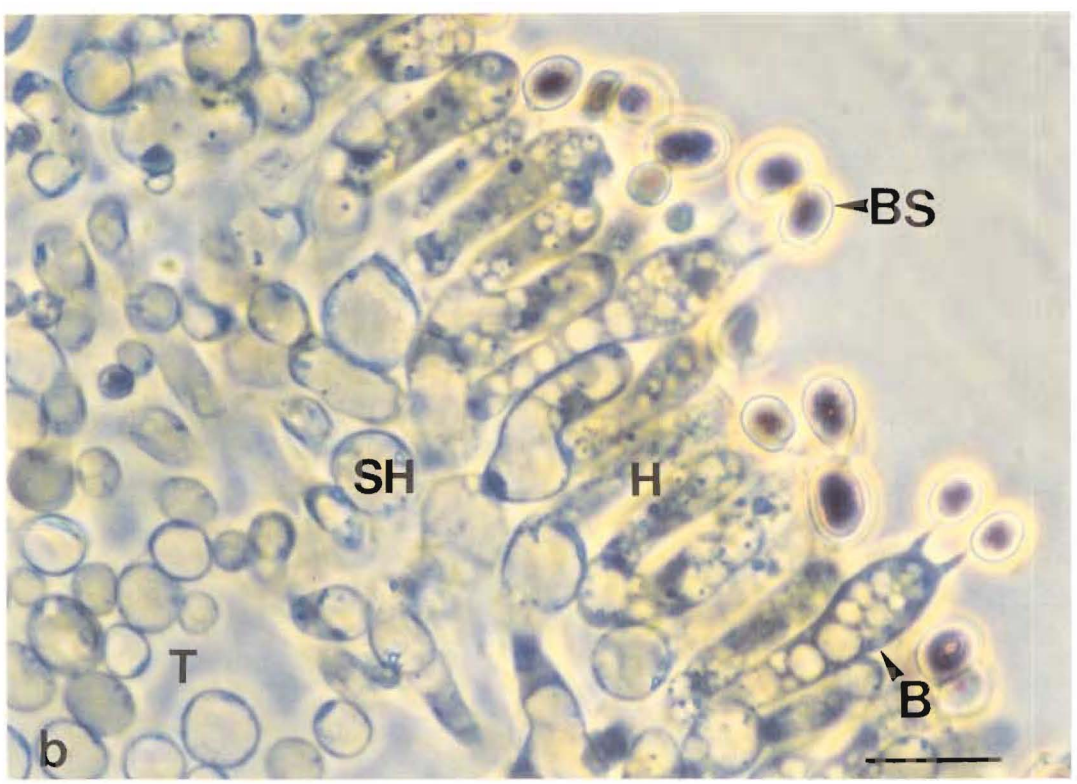
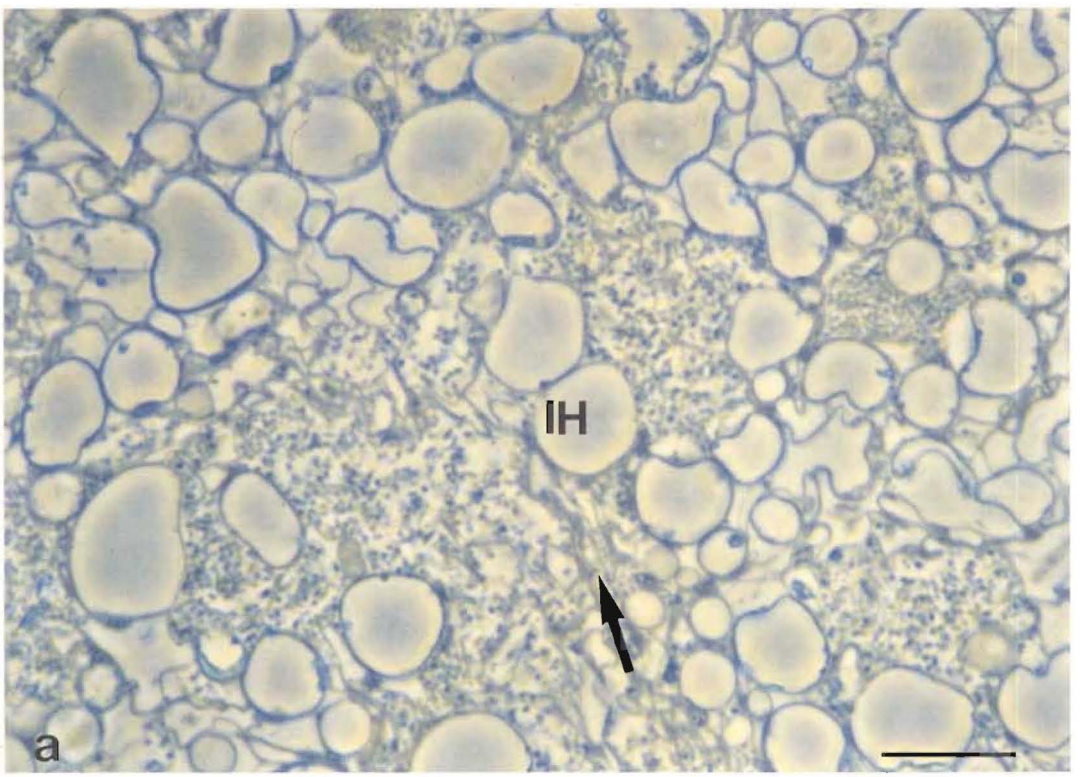

BS
SH
H
T
B
b

FIGURE 2.6

Fig. 2.6a 'Drippy gill' bacteria in *Agaricus* outer stipe tissue. The hyphae are loosely packed and the consequent large intercellular spaces are full of bacteria. Isolated hyphae (IH) remain intact within the 'stream' of bacteria, while areas of compressed hyphae are visible (arrow). Bar = 10μm.

Fig 2.6b *Agaricus* outer stipe tissue from a 'clean' sporocarp. Note the large intercellular spaces (IS) between loosely packed hyphae. A collapsed cell is indicated (arrow). Bar = 10μm


IH
a

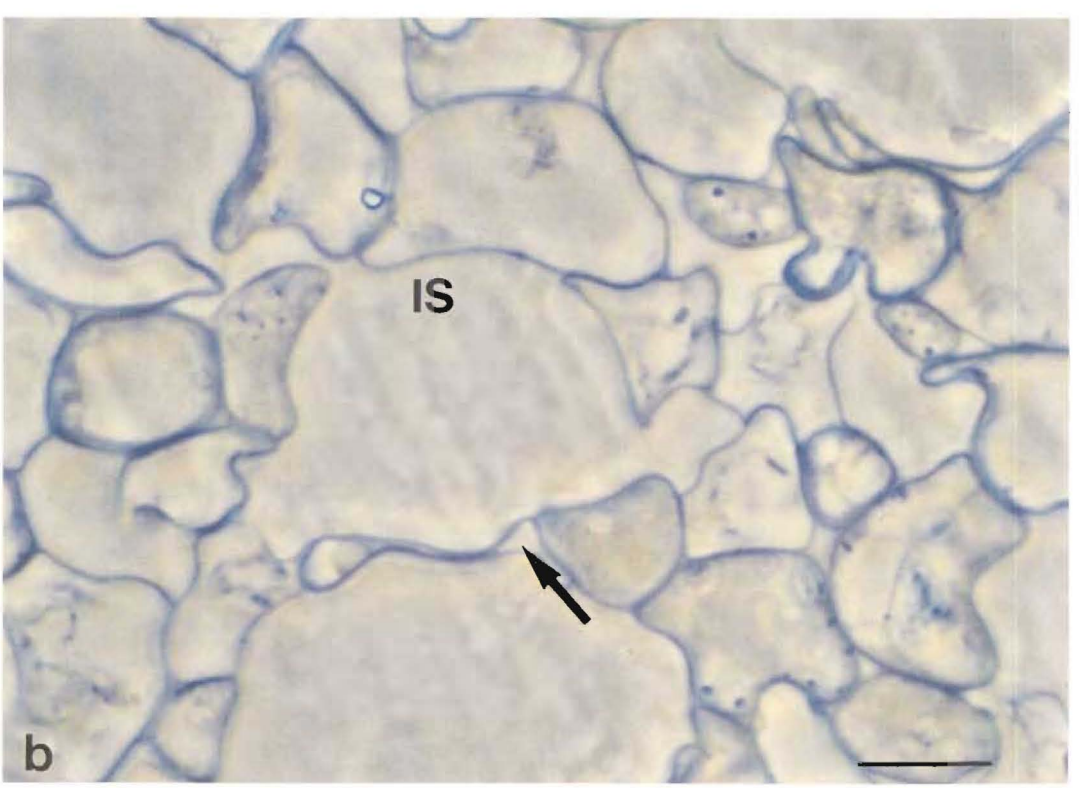
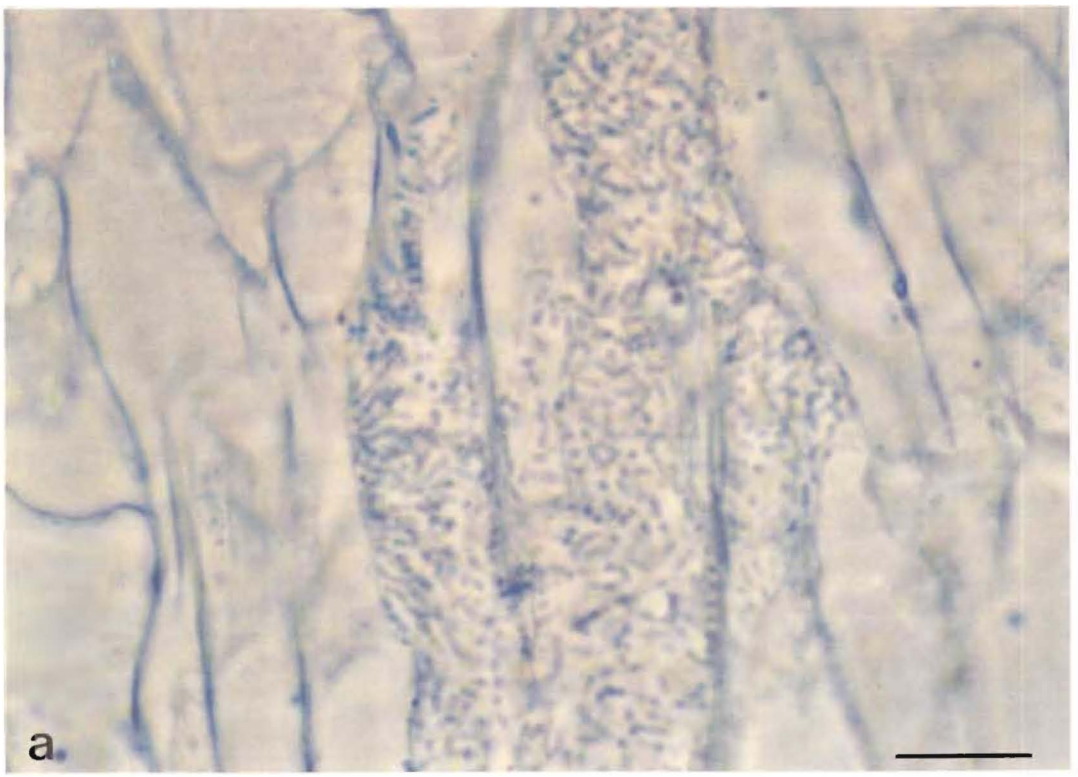

IS
b

FIGURE 2.7

Fig. 2.7a 'Drippy gill' bacteria in *Agaricus* outer stipe tissue in longitudinal section. Determining intrahyphal bacteria is difficult due to the hyphae weaving in and out of section. The cells are not vacuolate as shown in 'normal' stipe tissue. Bar = 10μm.

Fig 2.7b *Agaricus* outer stipe tissue from a 'clean' sporocarp in longitudinal section. Note the large intercellular spaces (IS) between loosely packed, vacuolate hyphal cells (VH). Bar = 10μm.


a.

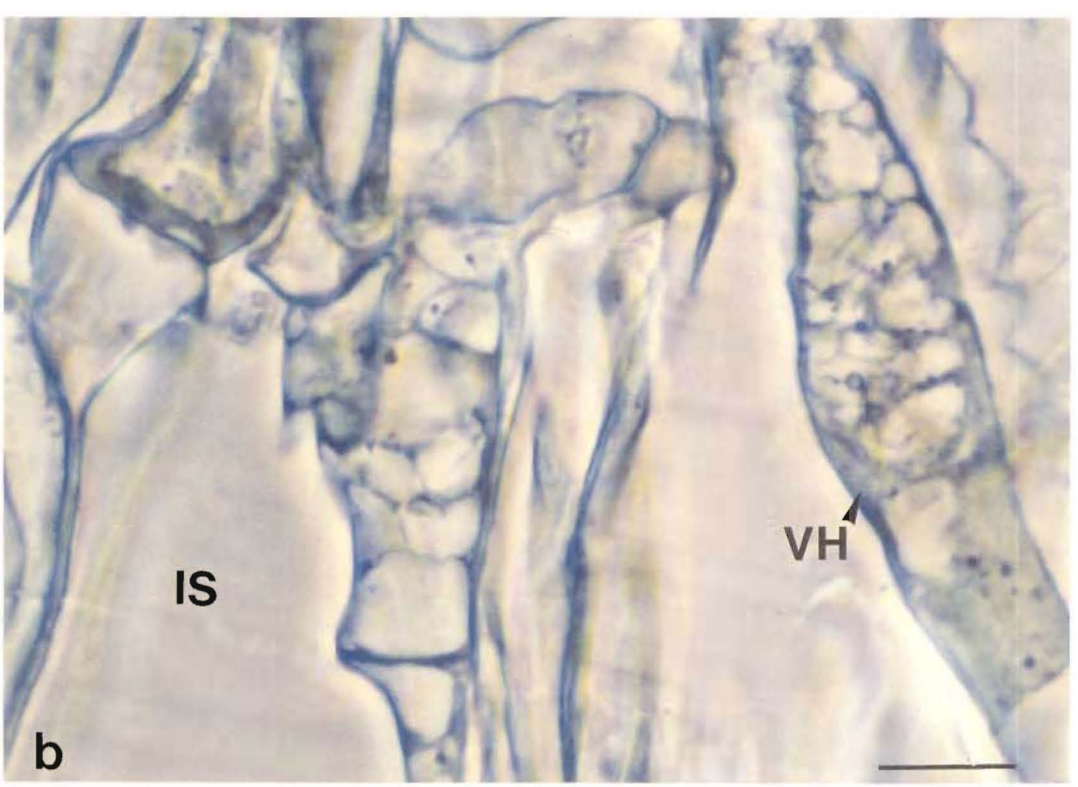
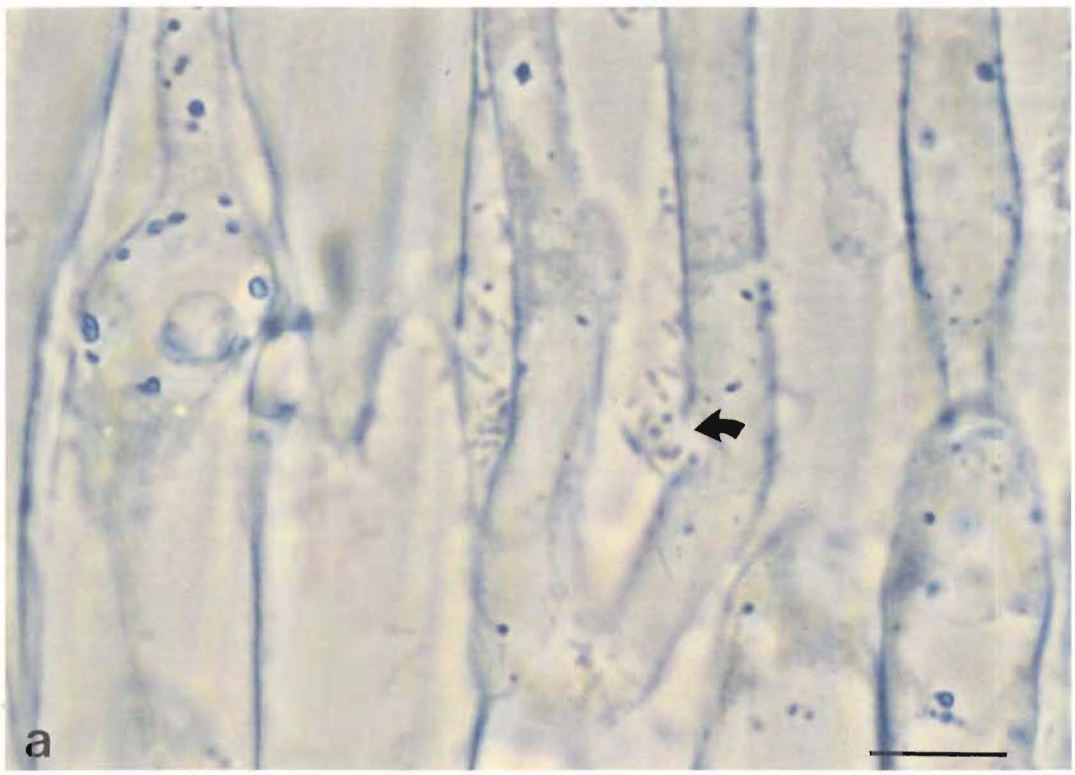

IS
VH
b

FIGURE 2.8

Fig. 2.8a 'Drippy gill' bacteria in *Agaricus* inner stipe tissue in longitudinal section. Bacteria are scarce in this tissue, but when present are again predominantly extrahyphal. Some bacteria in this section appear to be entering a hyphal cell through a broken wall (arrow). Bar = 10μm.

Fig 2.8b *Agaricus* inner stipe tissue from a 'clean' sporocarp in longitudinal section. Note the large intercellular spaces (IS) between loosely packed, vacuolate hyphal cells (VH). Bar = 10μm.

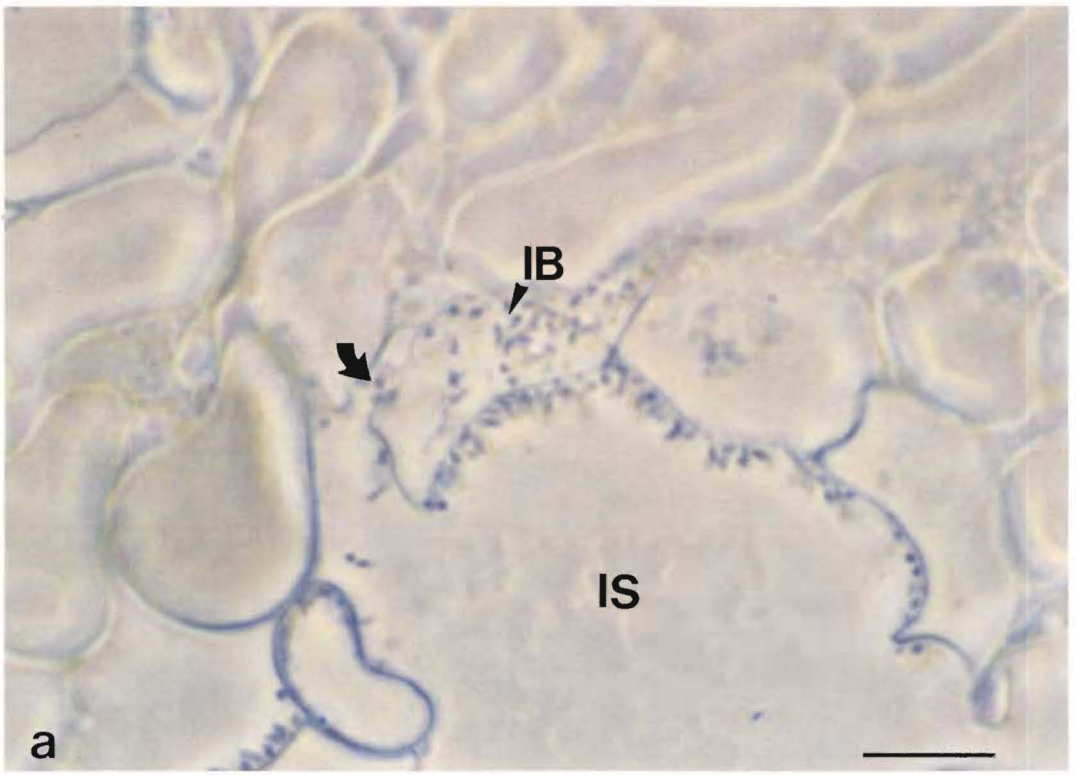

a


IS
VH
b

FIGURE 2.9

Fig. 2.9a 'Drippy gill' bacteria in *Agaricus* central cap tissue. Bacteria in the intercellular space (IS) are seen to attach themselves 'end on' to the fungal hyphal cells. Some intrahyphal bacteria (IB) appear to have gained entry through a broken hyphal wall (arrow). Bar = 10μm.

Fig 2.9b *Agaricus* outer stipe tissue from a 'drippy gill' affected sporocarp. This section demonstrates the large intercellular spaces which, when colonized, may lead to splitting of the stipe by the hyphae separating in the direction of the curved arrows. Broken hyphae, open to occupation by bacteria, are seen in different areas of the section (arrows). Few intrahyphal bacteria (IB) are visible. Bar = 10μm.


IB
IS
a

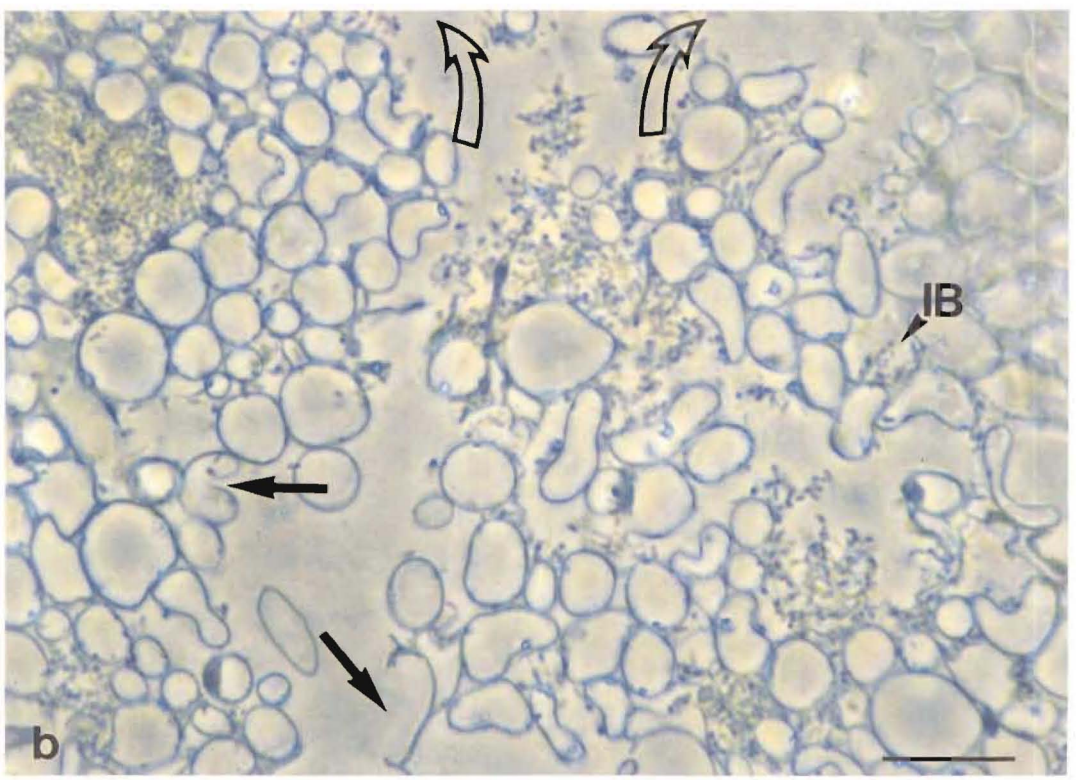

IB
b

FIGURE 2.10

Fig. 2.10a Extrahyphal 'drippy gill' bacteria in the stipe of an *Agaricus* sporocarp. Fungal cells showing healthy, highly vacuolate, organized cytoplasm (FC) appear to be unaffected by the bacteria. Bar=2μm.

Fig. 2.10b 'Drippy gill' bacteria in the stipe of an *Agaricus* sporocarp. The bacteria are predominantly extrahyphal. However, some individual cells may be intrahyphal (arrow), which demonstrates the difficulty of distinguishing between hyphal intercellular spaces and fungal cells. Bar=2μm.

Fig. 2.10c 'Drippy gill' bacteria in close proximity to an *Agaricus* stipe cell wall. There is no disruption of the fungal wall and the fungal cytoplasm (FC) appears to be intact and healthy. No evidence of adhesion can be seen. Bar=200nm.

Fig. 2.10d 'Drippy gill' bacterium against an *Agaricus* cell wall. It seems this bacterium has elicited a swelling from the fungal cell wall (FW). Again there is no evidence of adhesion. Bar=200nm

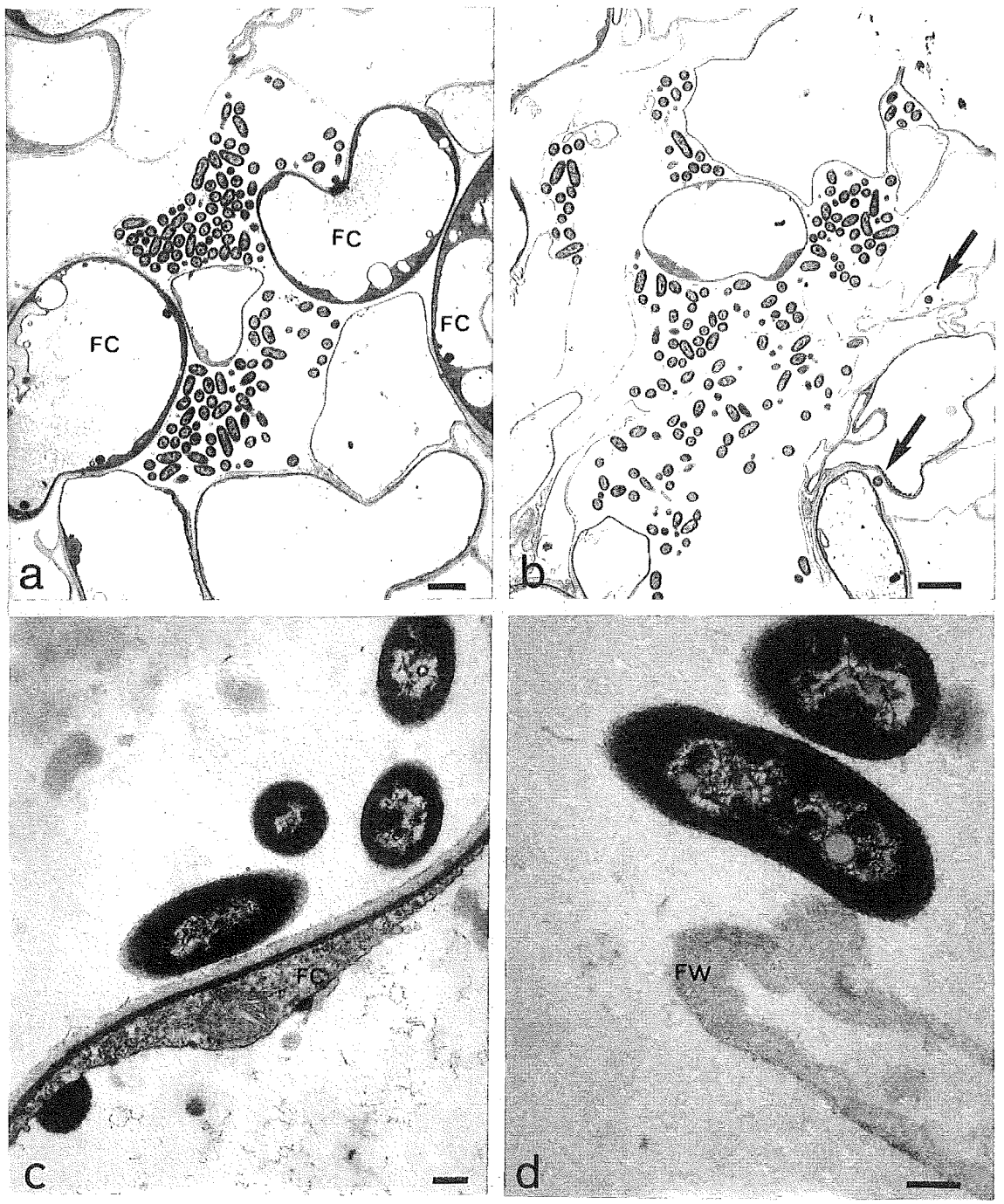

FC
FC
FC
a
b
FC
c
FW
d

FIGURE 2.11

Fig. 2.11a Extrahyphal 'drippy gill' bacteria in the hymenium of an *Agaricus* sporocarp. The bacteria appear to be congregating at the entrance of a wall break (arrow). Note the healthy, unaffected fungal cell (FC) in close proximity to the bacteria. Bar = 500nm.

Fig. 2.11b *Agaricus* hyphal wall artefact. The 'wall break' appears to be an aberration caused by a collapsed fungal cell folded on itself, giving the appearance of a wall discontinuity. This demonstrates the difficulty in identifying true breaches in the fungal cell walls. Bar = 100nm.

Fig. 2.11c Extrahyphal 'drippy gill' bacteria in the cap of an *Agaricus* sporocarp. The bacteria surround apparently healthy fungal cells. Note the absence of the extracellular matrix in the immediate vicinity of the bacterial cells. Intact remnants of the matrix can be seen (arrows). Bar = 1μm.

Fig. 2.11d 'Drippy gill' bacteria in an *Agaricus* sporocarp cap. The extrahyphal bacteria in the smaller group (arrow) are difficult to distinguish as such, requiring careful examination of the surrounding fungal cell walls. Bar = 1μm.

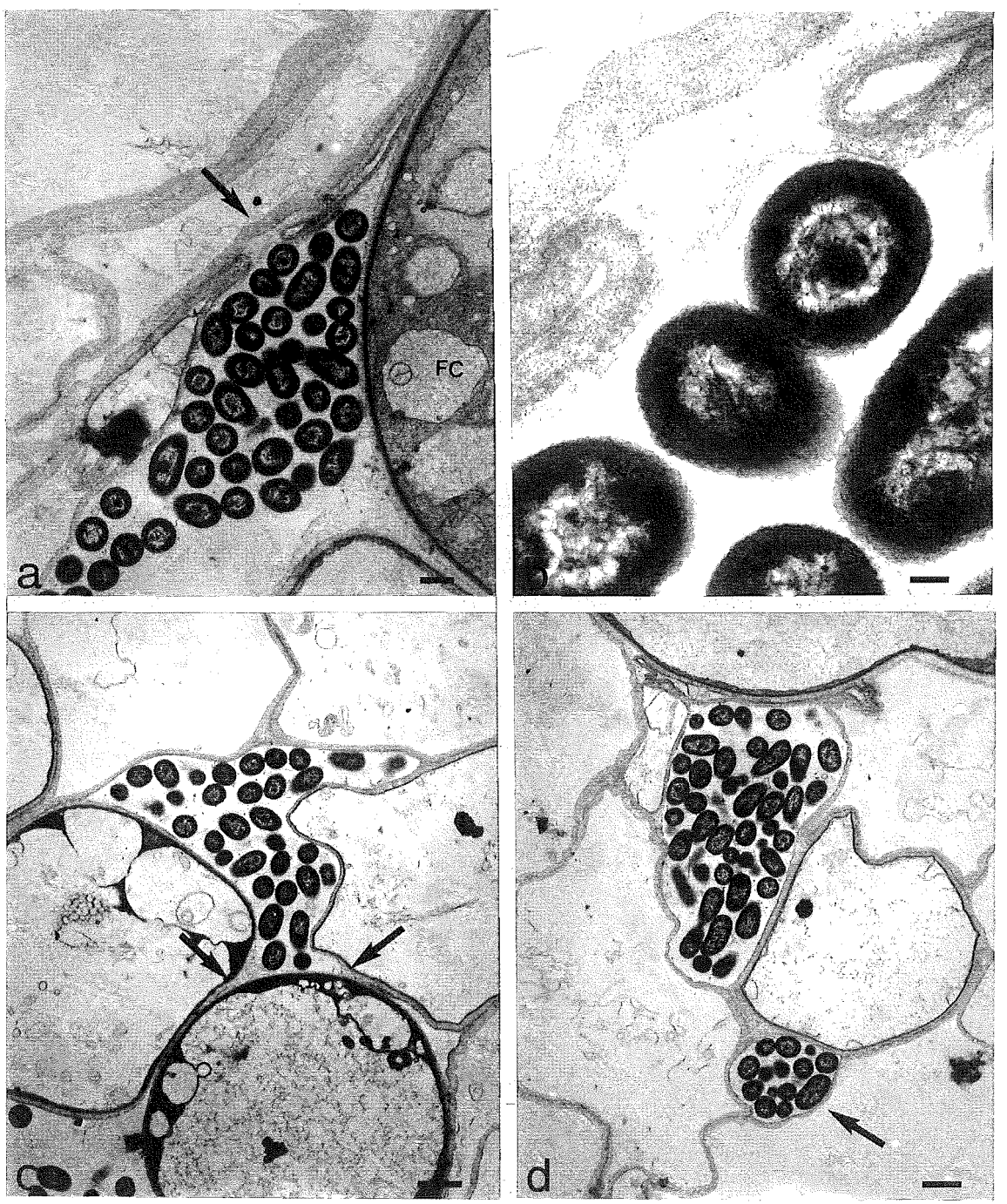

FC
a
b
c
d

FIGURE 2.12

Fig. 2.12a Extrahyphal 'drippy gill' bacteria in the cap of an *Agaricus* sporocarp. Note the healthy, intact fungal cells surrounding the bacteria. There is evidence of bacterial adhesion to the fungal wall (arrow) and a disruption to the outer fungal glucan layers (curved arrow). Bar=500nm.

Fig. 2.12b 'Drippy gill' bacteria passing through a broken hyphal wall. It is impossible to discern from this if the bacteria actively degrade the wall or are exploiting a pre-existing break. Note the profusion of microfibrils (M), presumably bacterial flagella, in the intercellular space (IS). Bar=500nm.

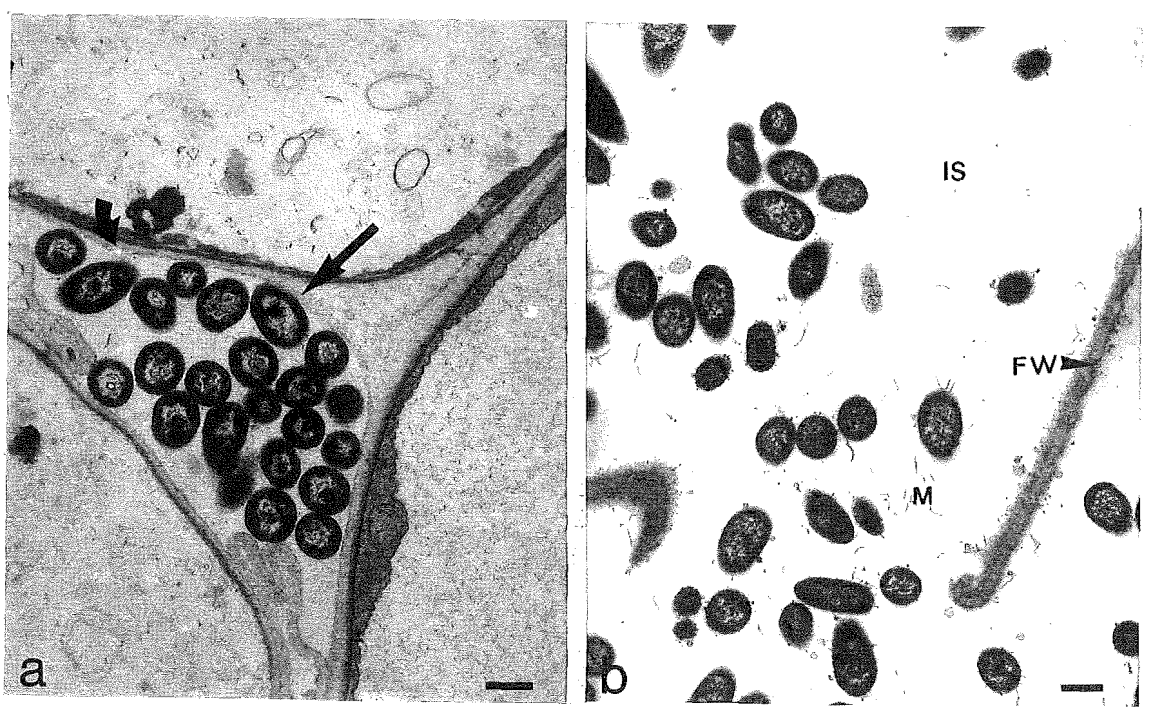

a
IS
FW
M
b

FIGURE 2.13

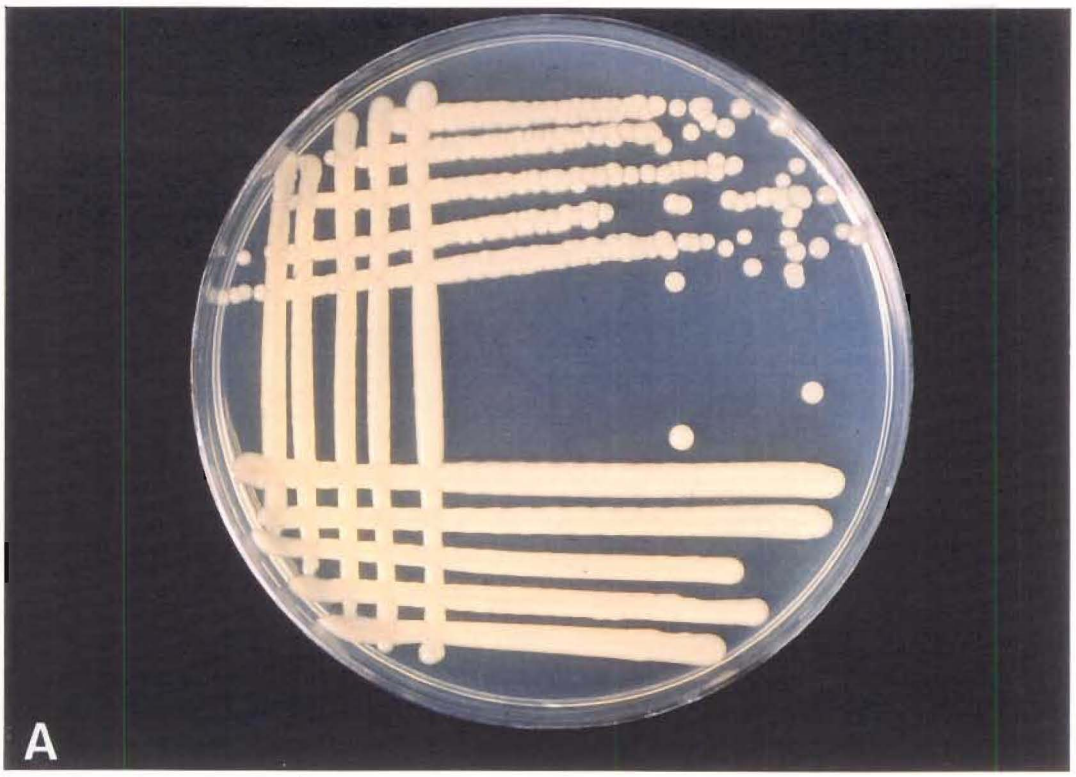
Fig. 2.13 A bacterium entering an *Agaricus* hyphal cell. Note the microfibrils presumably released from the hyphal wall (arrowhead). The bacterial outer envelope appears smooth unlike the crenulate outer membrane normally associated with 'drippy gill' bacteria. The intrahyphal bacteria appear to be surrounded by an electron dense amorphous material. Note also the emerging membrane at the point of entry (arrow), the flagellar remnant (FR) and coiled structures (CS). Bar=200nm.


FR
CS

FIGURE 2.14

Fig. 2.14a 'Drippy gill' isolate PMS601 (Type Strain) streaked on KB agar. Note the lack of smooth to rough transformation in the colony morphology.

Fig. 2.14b Isolate PV29 streaked on KB agar. Note the lack of smooth to rough transformation in the colony morphology.


A

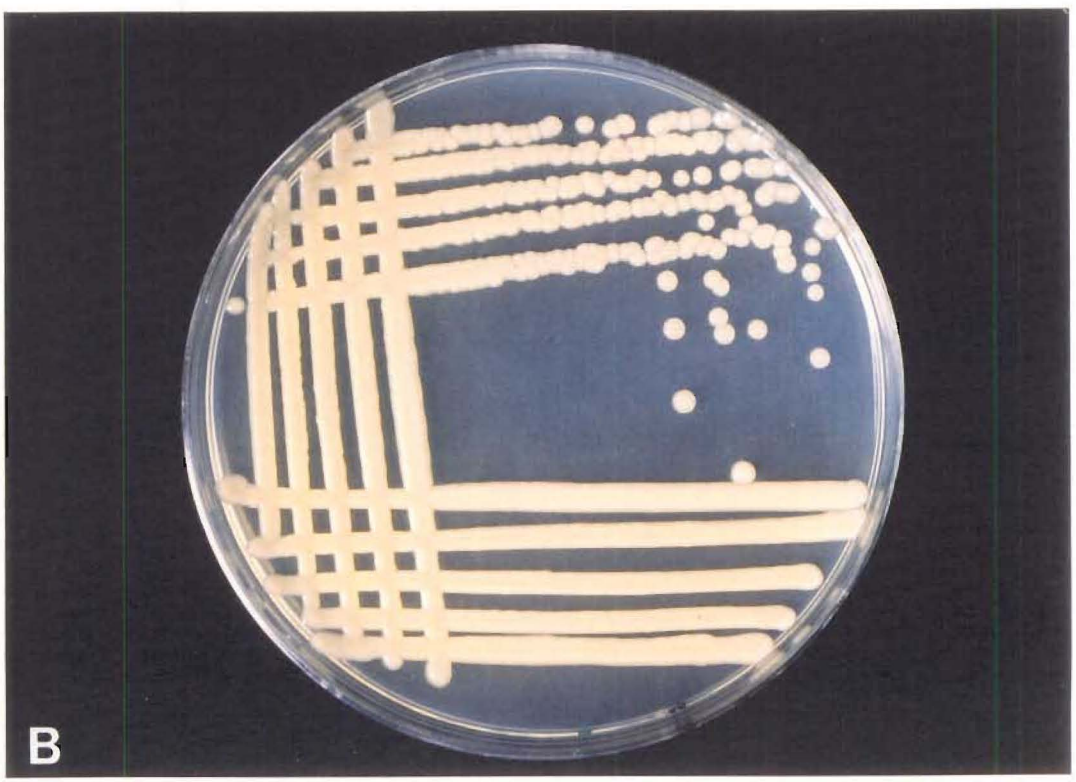

B

FIGURE 2.15

Fig. 2.15a 'Drippy gill' isolate PMS601 (Type Strain) inoculated into a tobacco leaf.

Fig. 2.15b 'Drippy gill' isolate PMS603 inoculated into a tobacco leaf.

Fig. 2.15c 'Drippy gill' isolate PMS752 inoculated into a tobacco leaf.

Fig. 2.15d Isolate PV29 inoculated into a tobacco leaf.

Fig. 2.15e Tobacco hypersensitive reaction positive control: PMS763 (*Pseudomonas syringae* pv *phaseolicola*) eliciting a hypersensitive response from a tobacco leaf.

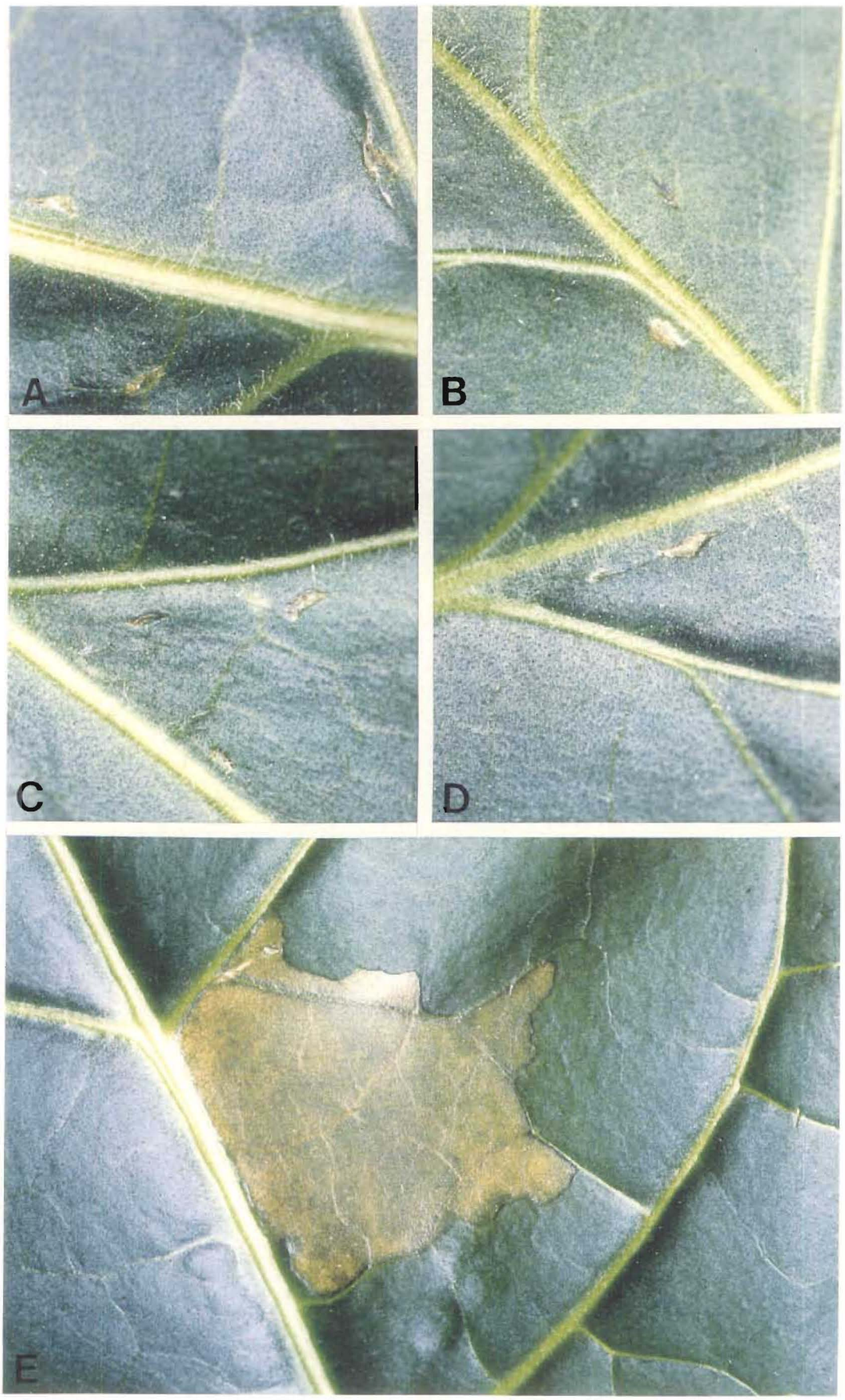

A
B
C
D
E

FIGURE 2.16

Fig. 2.16a Rifampicin resistant 'drippy gill' isolate PMS601 (Type Strain) from *Agaricus* hymenium growing on NA augmented with rifampicin.

Fig. 2.16b Rifampicin resistant 'drippy gill' isolate PMS603 from *Agaricus* hymenium growing on NA augmented with rifampicin.

Fig. 2.16c Rifampicin resistant 'drippy gill' isolate PMS752 from *Agaricus* hymenium growing on NA augmented with rifampicin.

Fig. 2.16d Rifampicin resistant isolate PV29 from *Agaricus* hymenium growing on NA augmented with rifampicin.

Fig. 2.16e Rifampicin control plate. The growth is due to fungal contaminants on the *Agaricus* sporocarp.

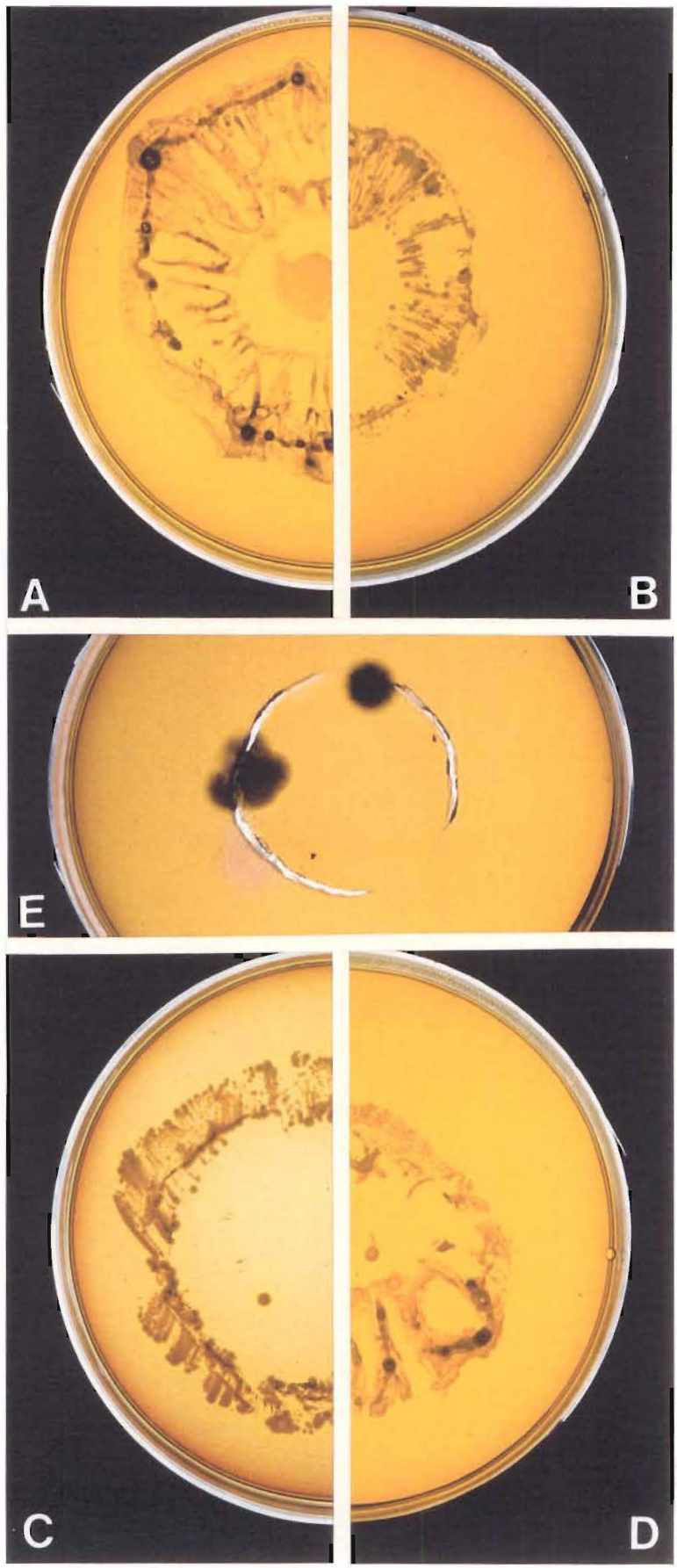

A
B
E
C
D

FIGURE 2.17

Fig. 2.17a PV29 toxin assay. Note the increased growth of *Agaricus* mycelium over the bacterial streak.

Fig. 2.16b PMS601 toxin assay. The bacterium seems to have no visible effect on the growth of *Agaricus* mycelium.

Fig. 2.16c PMS603 toxin assay. The bacterium seems to have no visible effect on the growth of *Agaricus* mycelium.

Fig. 2.16d PMS752 toxin assay. The bacterium seems to have no visible effect on the growth of *Agaricus* mycelium.

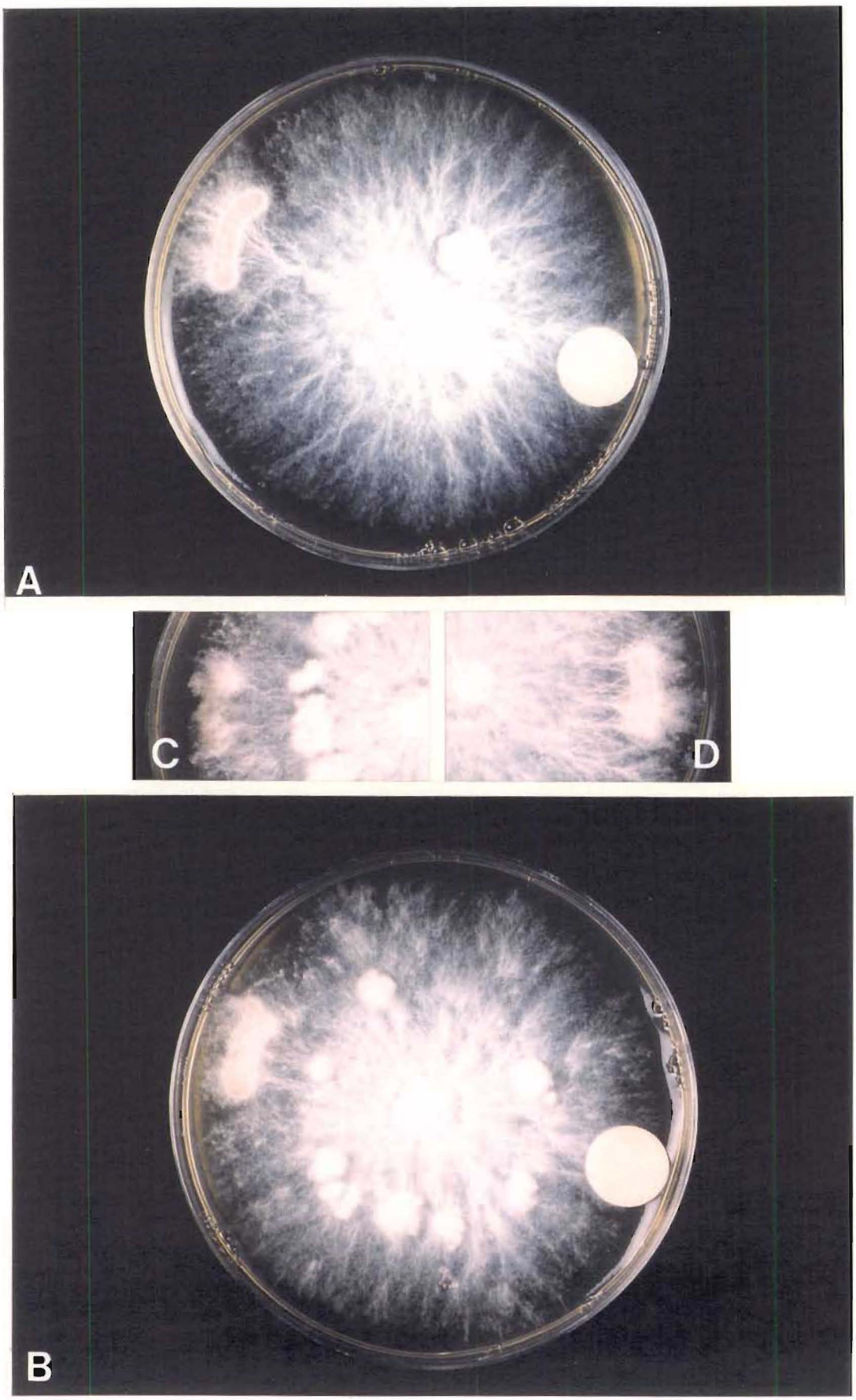

A
C
D
B

FIGURE 2.18

Fig. 2.18a 'Drippy gill' bacteria on *Agaricus* vegetative mycelium. Note the 'end on' attachment of the bacteria to the hyphae. Bar = 5μm.

Fig. 2.18b Intrahyphal 'drippy gill' bacteria in *Agaricus* mycelium. The septum between the cells (arrow) appears to be intact. Bar = 5μm.

Fig. 2.18c 'Drippy gill' bacteria on *Agaricus* mycelium. They tend to aggregate around the hyphae, although no 'end on' attachment is visible. Bar = 5μm.

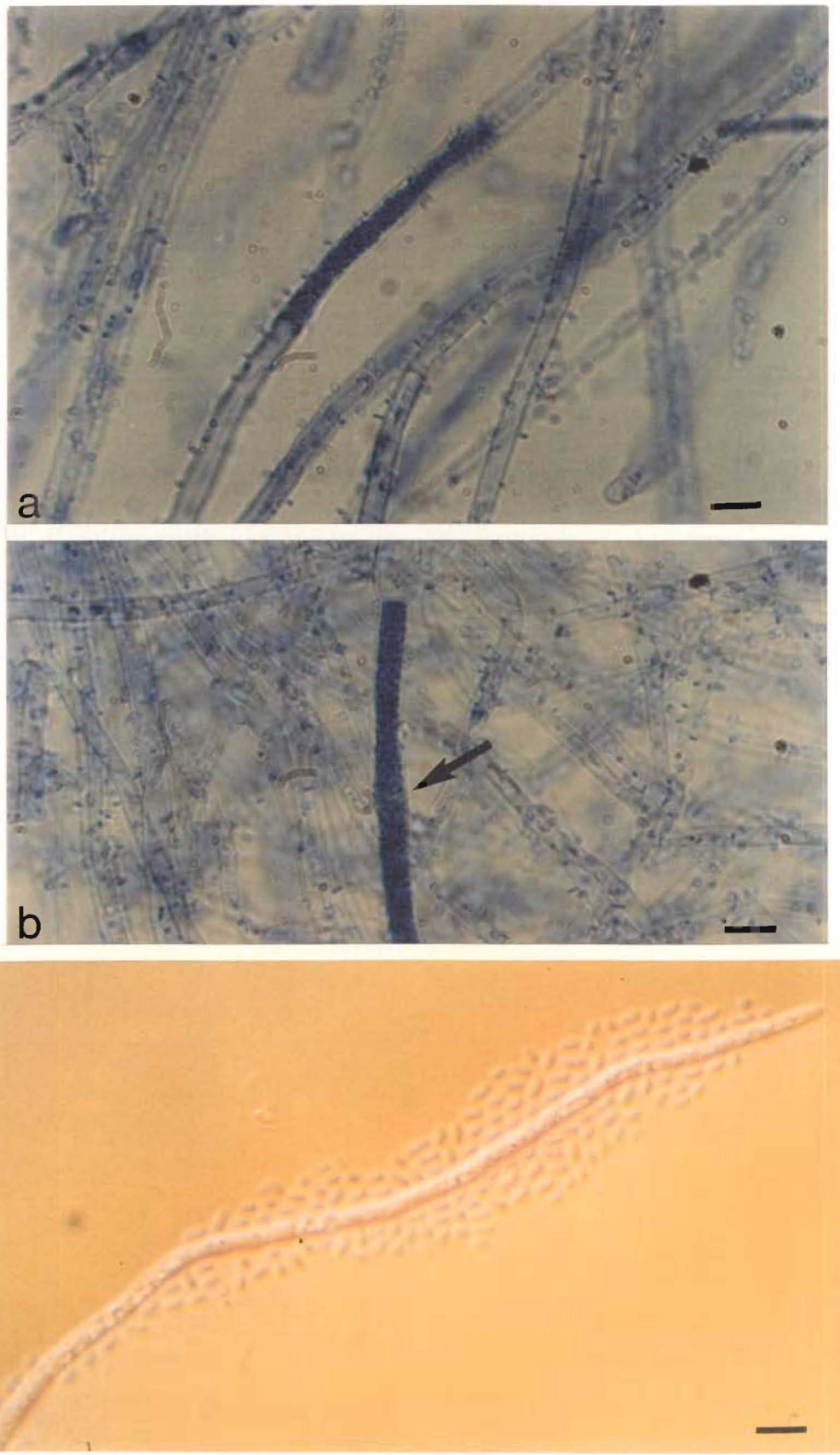
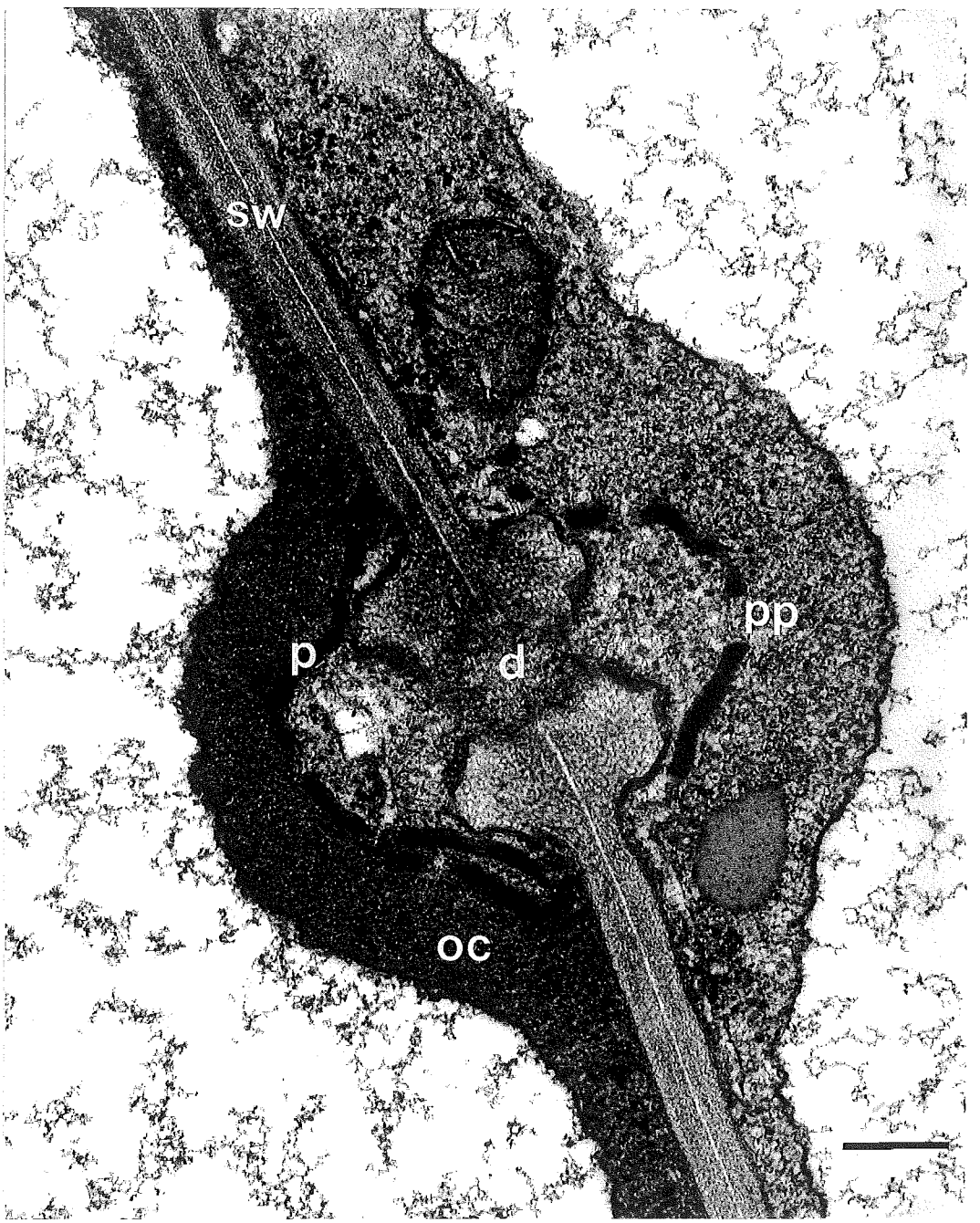

a
b
c

FIGURE 2.19

Fig. 2.19 The dolipore parenthesome septum. This presents a physical barrier to bacterial intrahyphal transmission and consists of the dolipore (d), the parenthesome (p), the parenthesome pores (pp), the septal wall (sw) and the outer cap (oc). Bar = 200nm.


sw
p
d
pp
oc

2.4 DISCUSSION

'Drippy gill' symptoms on *Agaricus* sporocarps from the natural outbreak investigated closely parallel the description of the disease given by Young (1970). However, cap distortion and retarded hymenial development were not evident.

In all tissues examined microscopically 'drippy gill' bacteria were observed extrahyphally. The intrahyphal occurrence of the causal organism was rare. Even in the presence of the bacteria, pileal and peripheral tissues retained their organization and hyphal cells appeared unaffected. In the hymenium, the trama and hymenial layers were indistinct and the tissue integrity disrupted. Turgid, vacuolate basidia were rarely seen in the immediate vicinity of the bacteria. The inner stipe tissue remained largely uncolonized by 'drippy gill' bacteria, however, the outer stipe showed profuse bacterial infection. The outer stipe hyphae showed a decrease or loss in vacuoles. The degree of vacuolation is variable throughout the Basidiomycete stipe (Craig *et al* 1979) so the significance of this observation remains obscure. Control tissue may not have been extracted from exactly the same region of the stipe as the 'drippy gill' affected tissue and thus comparison between the two tissues in this respect may be questioned.

Outer stipe hyphae and hence the large intercellular spaces are orientated approximately parallel to the longitudinal stipe axis (Angeli-Papa and Eyme 1978; Craig *et al* 1979). The degradation of the stipe extracellular matrix results in hyphal separation as suggested by Skellerup (1984) and longitudinal splits develop, exposing the bacteria-coated inner surfaces. In severe cases, these splits pervade the inner stipe tissue. The removal of the extracellular matrix from the hymenial and tramal hyphae results in the loss of integrity of the gill lamellae. As this tissue is external, there is nothing to prevent the hyphae separating and the tissue collapsing, as it appears to do.

'Drippy gill' bacteria failed to produce a toxin active against *Agaricus* mycelium both in plate assay and as a cell-free culture filtrate applied to tissue blocks. This observation was reinforced by TEM micrographs which

failed to show modifications to the fungal cytoplasm when bacteria were in close proximity to the hyphae. The two mushroom blotch causal organisms *P. tolaasii* (Skellerup 1984) and *P. gingeri* (Nott 1989) have been shown to elicit a detrimental response from *Agaricus* sporocarps, presumably in response to a toxin (Nair and Fahy 1973), for example tolaasin (Brodey *et al* 1991). The similarities of the reactions of mushroom hyphae to both these organisms suggests that *P. gingeri* also produces a toxin (Nott 1989). The detrimental reaction results in a 'plug' of modified or dead hyphae below the point of infection which prevents sporocarp invasion by the bacteria. Because 'drippy gill' bacteria do not stimulate such a reaction, access to the sporocarp is not blocked and bacteria are able to infiltrate the pileus.

'Drippy gill' symptoms are expressed on *Agaricus* sporocarps following the inoculation of *P. agarici* and PV29 onto the cap surface. The bacteria are able to penetrate the mature pileipellis, (the upper 'skin') and progress through the pileus to the hymenium and the stipe. It seems, therefore, the bivangeliocarpic development would provide scant protection against 'drippy gill' bacteria. That symptoms are expressed before veil-break (Young 1970; Rainey and Cole 1988) is not surprising, given the ability of the bacteria to penetrate and pass through protective membranes.

The causal bacteria are seen to 'attach' to fungal hyphae 'end on' in a similar manner to that described for *P. putida* by Rainey (1989). He also noted the intracellular occurrence of the non-pathogenic *P. putida* and concluded that entry to mushroom hyphae was via a broken fungal wall. The possibility of intrahyphal transmission of the bacterium was not discussed, but it appears very similar to the situation seen in 'drippy gill'. The dolipore parenthesome septum presents a physical barrier to bacterial intrahyphal transmission, particularly in the subhymenium, where it possesses a further obstruction, an outer cap (Gull 1976). As the existing pores are too small to allow the passage of bacteria and assuming the intrahyphal bacteria target the dolipore parenthesome septum, they must possess a suitable enzyme complement, chitinase and a ß-glucanase (Janszen and Wessels 1970), to dissolve the septum. Considering the structure of the dolipore parenthesome septum, enzymic dissolution would be a major undertaking. Bacteria relying on intrahyphal transmission may

target the septum surrounding the parenthesome and bypass the complex structure completely.

Evidence was obtained showing a bacterium actively entering a hyphal cell (Fig. 2.13). Bacteria have been demonstrated passing into hyphal cells via a supposedly mechanical break. Bacterial invasion, if it is to be a prerequisite for disease expression, must occur at a greater rate than observed.

Artificial inoculations show that the *P. agarici* and PV29 isolates induce identical symptoms on *Agaricus* sporocarps under the same conditions. The bacteria were confirmed as causal organisms of 'drippy gill' syndrome by satisfying Koch's postulates. Of the limited number of biochemical amd nutritional tests completed, all yielded the same results for the *P. agarici* bacterial isolates and PV29, which suggests these bacteria are the same species. The tests which Rainey and Cole (1988) used to differentiate PV29 from *P. agarici* yielded identical, negative results for both organisms. As a consequence, it appears their proposal to create a subdivision of LOPAT group III to accomodate PV29 is unwarranted. The variable tobacco hypersensitivity results of Bateson *et al* (1972) were not reproducible in this study, though the true identity of their isolates is questionable (Fahy 1981). That 'drippy gill' bacteria should be assigned to LOPAT group III is also questionable. Group III members have a LOPAT classification of - + - - + as does *P. cichorii* (Lelliott *et al* 1966; Fahy and Lloyd 1983). The absence of tobacco hypersensitivity precludes *P. agarici* from group III and a new group should be created. *P. agarici* is recognised as forming a discrete phenon distinct from all other fluorescent pseudomonads (Fahy 1981) which should be reflected in the LOPAT system, which, though perhaps outdated, is still very useful. The variable responses of 'drippy gill' bacteria to a range of antibiotics lend credence to the suggestion that this test should be used as a diagnostic aid rather than a taxonomic criterion because of the possibility of mutation and consequent change in response (Gilardi 1971).

P. agarici is an interesting case in terms of pseudomonad colony transformation. *P. tolaasii* and *P. gingeri* lose their toxin producing ability when they transform from the smooth to rough colony morphology (Cutri

et al 1984). In the rough form, *P. tolaasii* has been shown to behave similarly to the saprophytic *P. putida* even as far as stimulating mycelial growth (Rainey 1989). *P. agarici* does not sector as readily as *P. tolaasii* and the rough colonial type is difficult to isolate in pure culture. The 'fried egg' morphology, dismissed as being a mixed culture by Cutri *et al* (1984), was considered an example of reversion of rough to smooth (Olivier and Guillaume 1981 in Cutri *et al* 1984), an event assumed to occur but not observed in plate culture. *P. agarici* does not produce a toxin, and indeed the smooth, pathogenic colony morphology has been shown to stimulate mycelial growth in the same manner as the saprophytic *P. putida* and the rough *P. tolaasii* variant. It may be possible, therefore, for *P. agarici* to lose its virulence without undergoing the characteristic smooth to rough transformation. *P. agarici* may represent an important intermediate in the question of transformations and the origins of pathogenesis and consequently merits further work.

Growth room trials indicated that the greatest rate of 'drippy gill' infection was achieved from inoculated casing, a finding consistent with that of Young (1970) investigating 'drippy gill' and Wong and Preece (1980) in the case of *P. tolaasii*. That the compost treatment yielded so few diseased sporocarps supports the suggestion that the causal organism is not intrahyphal. In the compost, hyphae are exposed to the bacteria over a number of weeks, allowing for hyphal infection and subsequent symptom expression following emergence of the sporocarps from the casing. The fact that this does not occur implies that the early formed fruit body is required for infection. Maximum infection occurs when bacteria are introduced to formed and developing sporocarp initials in the casing layer. The outer protective membrane has been shown to be permeable to 'drippy gill' bacteria.

Water borne inoculum may perhaps build up a sufficiently large inoculum on the growing bed over a longer time frame than required to produce three or four flushes. A water sample removed from a commercial mushroom farm at the time of an outbreak, however, failed to show any sign of *P. agarici*, casting doubt on water as a primary source of infection.

CHAPTER THREE

ULTRASTRUCTURAL STUDIES OF 'DRIPPY GILL'

3.1 INTRODUCTION

3.1.1 The Fungal Wall

The fungal cell wall, as an interface between the fragile cytoplasm of the organism and its environment, must perform a number of vital functions. It is not merely a metabolically inert shell protecting a delicate cytoplasm (Farkas 1979), rather it is a dynamic organelle carrying out a diverse array of physiologically crucial functions, such as controlling the tenuous cytoplasmic osmotic balance by restricting water content (Peberdy 1990). It possesses the ability to monitor the secretion and uptake of molecules other than water into the cell, thus regulating the nutrition and metabolism of the organism, as well as providing a store of carbon reserves demonstrated by the conversion of wall glucans into glucose by endogenous glucanases, particularly in glucose-depleted environments (Sietsma *et al* 1977).

Fungal cell walls also play a major role as a physical barrier, protecting the cytoplasm from many external hazards such as exogenous lytic enzymes, particularly chitinases and glucanases (Bull 1970; Garcia Mendoza *et al* 1979) and UV radiation, τ radiation and X-rays (Zhadnova *et al* 1973 in Peberdy 1990). It confers shape and rigidity to the mature cell while allowing expansion both apically, as in a growing vegetative hypha, or by diffuse extension as required by hyphae in some fruit bodies (Wessels *et al* 1990). It also supplies the organism with critical cell-to-cell interactions, particularly important should the fungus' environment be another organism (Peberdy 1990), as in the case of a parasite or symbiont.

The fungal cell wall because of its construction, componentry and function, distinguishes the fungi from other living organisms (Bartnicki-Garcia 1968). It is a complex array of interacting compounds which nourish and protect the organism. In order to actively breach the cell wall and penetrate the sensitive cytoplasm, mycopathogenic bacteria must possess a complement of enzymes capable of degrading the wall. By understanding the chemistry and organization of fungal walls, the mechanisms of bacterial pathogenesis may become clearer.

3.1.2 *Agaricus* Wall Chemistry and Ultrastructure

Fungal cell walls are characteristically composed of polysaccharides (constituting 80-90% (w/w) of wall material) and minor components such as proteins, lipids and pigments. The close correlation between cell wall chemistry and the taxonomic subdivision of fungi has been recognized by Bartnicki-Garcia (1968), who was able to group fungi according to the major polysaccharides present in their walls. The eight chemo-taxonomic groups thus created by Bartnicki-Garcia closely parallel the accepted taxonomy based on more traditional phylogenetic markers. The chemo-taxonomic group pertaining to the Basidiomycetes, Group 5, comprises fungi which possess chitin and glucan as their major wall polysaccharides and contains the Ascomycetes (except the Saccharomycetaceae), the Deuteromycetes (except the Cryptococcaceae and Rhodotorulaceae) and the Chytridiomycetes. While it is acknowledged that further detailed analyses of the minor wall polysaccharides and other less prevalent chemical constituents of the wall are required to validate a taxonomic system based on these properties (Ruiz-Herrera 1991), the similarities betwen the wall components of Group 5 member taxons are close enough to draw comparisons in terms of wall morphology and chemistry.

Michalenko *et al* (1976) proposed a model for *Agaricus bisporus* hyphal wall structure based largely on enzymatic degradation and chemical solubilization experiments. The more recent fractionation techniques which allow for the isolation and characterization of discrete wall fractions support their proposed model. They considered the *Agaricus* wall to consist of an internal layer adjacent to the plasmalemma, composed of chitin microfibrils embedded in a ß-(1,3) glucan matrix. Situated immediately to the outside of this is a loose, discontinuous layer of α-glucan which is enclosed in an amorphous ß-glucan mucilage. Protein is present, dispersed throughout the layers and does not appear as a separate and distinct layer as suggested by Hunsley and Burnett (1970) from their study of the Basidiomycete, *Schizophyllum commune.*

Novaes-Ledieu *et al* (1987) were able to isolate and characterize six distinct fractions from hyphal walls of *Agaricus bisporus* vegetative mycelium utilizing standard chemical treatments. These fractions and their chemical composition are summarized in Table 3.1. Fraction I comprises a water-soluble mucilage which is loosely bound to the outer wall surface and seemingly corresponds to the extensive extracellular matrix evident in mature sporocarps. Fraction II is composed largely of α-(1,3) glucan (alkali-soluble or 'S'-glucan) which exhibits a rodlet configuration at the outer wall surface. These researchers presumed the alkali-insoluble residue of fraction VI to comprise a network of chitin microfibrils embedded in an alkali-insoluble ß-glucan (or 'R'-glucan) matrix (thought by Sietsma and Wessels (1981) to be composed of ß-(1,3)-ß-(1,6) glucan).
In a similar study of *Armillaria mellea*, Sanchez Hernandez *et al* (1990) were able to isolate four distinct fractions from the walls of the vegetative mycelium as summarized below (Table 3.2). These workers analyzed the residual fraction (fraction IV) despite the high chitin content and confirmed the previous presumption of Novaes-Ledieu *et al* (1987) that the insoluble residue comprises both chitin (indicated by infrared absorption bands characteristic of the chitinous CO-NH linkage) and ß-(1,3) glucan.

Accepted chemical treatments of fungal walls result in an insoluble residue consisting of chitin and remnants of the wall's ß-glucan component. The indication of covalent linkages between these two skeletal polysaccharides in *Schizophyllum commune* (Sietsma and Wessels 1979) and *Agaricus bisporus* (Avellan *et al* 1986) was investigated further by Mol *et al* (1988) who demonstrated that depolymerization of the glucosaminoglycan results in solubilization and release of the majority of residual glucan. Subsequent alkali treatments release nearly all of the remaining glucan. The close association of ß-glucan and chitin microfibrils noted by Novaes-Ledieu *et al* (1987) and Sanchez Hernandez *et al* (1990) is thus explained by the chemical linkage between chitin and glucan.

	WALL FRACTION					
	I	II	III	IV	V	VI
% Wall (w/w)[a]	14.1	8.9	12.1	6.8	18.1	15.1
% Neutral Sugars	50.2	82.3	80.5	78.5	61.5	19.8
% Protein	38.7	5.8	7.9	9.9	5.2	2.8
% Glucosamine	3.2	t	t	t	22.0	61.2
Predominant Monomer	Glucose	Glucose	Glucose	Glucose	Glucose	-[b]
Predominant Linkage(s)	(1,4)	(1,3)	(1,3) (1,6)	(1,3) (1,6)	(1,3)	-[b]
Infra-Red Spectrum[c]	β	α	β	β	β	CO-NH[d]

[a] Percentage of wall material contained in isolated fraction
[b] Not determined due to chitin content of fraction
[c] Linkage type as determined by infrared absorption patterns
[d] IR spectrum typical of chitin bonds
t Trace

Table 3.1 Wall Chemistry of *Agaricus bisporus*

	WALL FRACTION			
	I	II	III	IV
% Wall (w/w)[a]	7.6	32.0	3.6	30.6
% Neutral Sugars	70.2	81.3	82.7	75.5
% Protein	25.0	13.5	8.5	1.2
% Glucosamine	0.1	0.2	0.2	19.7
Predominant Monomer	Glucose	Glucose	Glucose	Glucose
Predominant Linkage	(1,3)	(1,3)	(1,3)	(1,3)
Infra-Red Spectrum[b]	β	α	β	β + Chitin[c]

[a] Percentage of wall material contained in isolated fraction
[b] Linkage type as determined by infrared absorption patterns
[c] Two bands are present, indicating both chitin and β-linked glucose

Table 3.2 Wall Chemistry of *Armillaria mellea*

3.1.3 Chitin and Chitinase

Chitin, a long chained (1,4)-ß-linked homopolymer of N-acetylglucosamine (2-acetamido-2-deoxy-D-glucose) is a molecule of structural importance to a wide variety of organisms ranging from protists, to fungi, to invertebrates. Consequently, the annual production and steady state mass of chitin amounts to some 10^{10} to 10^{11} tons by estimation (Gooday 1990).

The polymer exists in three distinct forms:

A) α-chitin composed of antiparallel chains
B) ß-chitin composed of parallel chains
C) τ-chitin composed of a three chain unit cell

Of these, it is the α-chitin configuration which forms the skeletal component of fungal cell walls (Blackwell 1988) and animals (Lopez-Romero and Ruiz-Herrera 1985).

As chitin exists in so many different environments, there too exists a mirror distribution of chitinolytic organisms. Though the mode of action may be identical, the roles chitinase plays in natural systems are diverse. All chitinous fungi investigated, for example, have been shown to possess chitinases (Gooday 1990). As an autolysin, chitinase assists in spore release (Iten and Matile 1970) and is strongly purported to be a critical element in hyphal wall morphogenesis (Bartnicki-Garcia 1973) and branching (Mahadevan and Mahadkhar 1970). Many plants produce chitinase as a protection mechanism in response to fungal invasion (Boller *et al* 1983; Metraux and Boller 1986; Mauch *et al* 1988). Bacteria and fungi, able to produce chitinases exogenously, utilize the breakdown products of chitinous soil-borne substrates as a nutrient source.

More relevant to this investigation however, is the role chitinase plays in the pathogenesis by bacterial mycopathogens. As a consequence of its abundance, chitinases are produced by both Gram negative and Gram positive bacteria excluding the Archaebacteria (Gooday 1990). Pseudomonads are a recognized group of chitinase producers (Clarke and

Tracey 1956), being particularly abundant in estuarine waters (Reichardt *et al* 1983), freshwaters and soils (Sturtz and Robinson 1985; Gooday 1990).

In bacterial pathogenesis of fungi, chitinases are thought to play two major roles: penetration of the fungal wall, followed by the supply of nutrients as amino sugars from the degradation of chitin. Degradation of chitin is brought about by random cleavage of the polysaccharide by chitinase to form oligosaccharides, principally di-N,N'-acetylchitobiose, followed by degradation of the oligomers to N-acetylglucosamine. Chitinase does not appear to be the sole agent of chitin biodeterioration but is a complex of two enzymes, chitinase and chitobiase (Ohtakara *et al* 1978).

3.1.4 Glucan and Glucanase

Glucose is the most prevalent sugar in all fungal cell walls and largely occurs in the form of a homopolymer, glucan. Fungal glucans comprise the ß-glucans, which may be ß-(1,3)-linked without branching, or they may contain ß-(1,3) links with occasional ß-(1,6) branches.
Similarly, the α-glucans occur either as α-(1,3)-linked or alternating α-(1,3)/α-(1,4)-linked D-glucopyranosyl moieties and are microfibrillar in appearance, in the order of 10-14nm in diameter (Ruiz-Herrera 1991).

Glucans play an integral role in fungal structure and physiology in terms of their contribution to wall integrity and interhyphal adhesion as well as their function as stored reserve compounds. While commonly associated with fungal walls, glucans have also been identified as cytoplasmic inclusions in some Oomycetes, where they function as reserve material (Zevenhuizen and Bartnicki-Garcia 1970). In pathogenic fungi, glucans may play a part in surface recognition to susceptible hosts and may elicit a defensive response from the invaded host. While some glucans are seemingly confined to the fungal cell wall or within the fungal cytoplasm, other glucans are exuded into the medium where they accumulate as mucilage (Ruiz-Herrera 1991).
The extracellular matrix, most evident in the interhyphal spaces of the sporocarp stipe, is composed of glucan which performs several physiological and morphological functions. The amorphous/mucilagenous

nature of the extracellular matrix may act as a buffer, offering protection against adverse environments performing a role as a cryoprotectant or anti-desiccant. The matrix may also act as a transport mechanism, bathing hyphal cells in a nutrient reservoir, similar to the insect haemocoel. The mucilagenous character of the matrix enables adjacent hyphae to slide against each other whilst elongating at different rates. Perhaps of greatest interest with respect to the maintenance of sporocarp integrity is the adhesive property of the matrix, which binds adjacent hyphae together (Williams *et al* 1985).

To mobilize and activate the stored compounds in fungal walls and cytoplasm, a range of glucanases are produced by the fungus itself. As the most common glucan is ß-(1,3) glucan (laminarin), it follows that ß-(1,3) glucanase (laminarinase) is the predominant glucanase among the fungi. Other than mobilizing nutrients, glucanases, as autolysins, are also crucial in the development of the heterokaryotic state in the Basidiomycete life cycle. ß-(1,3) glucanase, in association with chitinase, assists in the dissolution of the fungal septum which allows nuclear migration (Giesy and Day 1965; Janszen and Wessels 1970), possibly by exposing chitin microfibrils from their protective β-glucan matrix, thus making them susceptible to degradation (Wessels and Marchant 1974).

3.1.5 Intrahyphal Bacteria

The occurrence of bacteria within fungal hyphae is not a new observation and examples can be seen throughout the fungi. It has long been thought the causal organism of mummy disease of the cultivated mushroom (*Agaricus bisporus*), first described by Tucker (1937, 1940) and subsequently by Tucker and Routien (1942), to be a bacterium which inhabits the hyphae of affected sporocarps (Schisler *et al* 1968; van Zaayen and Waterreus 1974; Kalbarczyk 1987; Betterley and Olson 1989). The bacterium has been described by these workers to be a pseudomonad and designated as a member of a sub-group of *P. fluorescens* biotype G by Betterley and Olson (1989). The mummy-causing pseudomonad remains distinct from both *P. tolaasii* and *P. gingeri*, which are also members of *P. fluorescens* biotype G.

Schisler *et al* (1968) were able to consistently isolate *P. tolaasii* from mummy-affected sporocarps. This is not unexpected since *P. tolaasii* is ubiquitous within the mushroom bed, it being a major component of the mushroom-associated microflora. Interestingly, Cole and Skellerup (1986), in their ultrastructural study of brown blotch of *A. bisporus* caused by *P. tolaasii*, noted the significant intracellular occurrence of the bacterium.

Intrahyphal subsistence is not confined to pseudomonads, nor indeed are intrahyphal bacteria restricted to *A. bisporus* and its relatives. Rickettsia-like organisms have been demonstrated within the hyphae of *Lentinus edodes* (Berk.)Sing. (Nakai and Ushiyama 1984). Investigations of these malformed fruitbodies revealed intracellular bacteria surrounded by seemingly healthy host cytoplasm containing intact nuclei, mitochondria and ground cytoplasm. This observation is in contrast to that of Kalbarczyk (1987) who described severe disruption of host cytoplasm and marked damage to the fungal cell wall in respect to intrahyphal pseudomonads in *Agaricus* mycelium.

While investigating the contact zone between *Armillariella mellea* and Pelargonium roots, Samyn *et al* (1980) reported the presence of bacteria between the fibre hyphae of *Armillariella* rhizomorphs and bacteria which were described as distinctly intracellular. Their findings suggest the intracellular bacterium to be a pseudomonad.

Attafuah and Bradbury (1989) isolated a bacterium from the mealybug *Planococcoides njalensis* to which they ascribed the name *Pseudomonas antimicrobica*. Their investigations into the antifungal activity of this bacterium revealed not only its apparent inhibition of mycelial growth of various test cultures, but more significantly, when the bacterium was inoculated onto a test plate containing *Phytophthora palmivora* (a pathogen of cocoa) subsequent microscopic examinations showed the advancing hyphal apices to contain bacterial cells. The invaded mycelium, when transferred to a fresh medium, did not grow. Although these workers recorded the intrahyphal appearance of the isolated bacterium within other test fungi, the viability of those invaded fungi was not reported.

The nature of 'drippy gill' symptoms has led people to believe the causal organism may exist and be transmitted intrahyphally. If this is the case, the bacteria must possess certain properties which allow for hyphal entry. This chapter reports on the investigation of the bacterial/fungal interaction, in particular, hyphal wall penetration by *P. agarici*.

3.2 MATERIALS AND METHODS

A] CHITINASE ASSAY

The ability of drippy gill isolates to produce the enzyme chitinase was evaluated utilizing the following methods.

3.2.1 Petri Dish Assay

3.2.1.1 Preparation of Chitin

Chitin was purified and prepared following a protocol supplied by Lorraine Bolger, Department of Plant and Microbial Sciences, University of Canterbury (pers. comm.). Chitin plates were then prepared as an overlay on a nutrient agar base.

1. 5g chitin (practical grade, Sigma Chemical Co.) were dissolved in 200ml of 85% phosphoric acid for 72h at 4^{o}C.
2. The dissolved chitin was then precipitated with approximately 2.5l distilled water. Water was decanted off and the washing procedure repeated 2-3 times.
3. The pH of the chitin suspension was adjusted with concentrated NaOH (pellet form) to the desired pH (6.8), washed again with distilled water 3-4 times, then centrifuged when the volume of chitin had reduced.
4. The chitin pellet was resuspended in phosphate-citrate buffer (0.075M $NaH_2PO_4.2H_2O$ / 0.075M $Na_3C_6H_5O_7.2H_2O$ pH6.8) to a concentration of 1% ensuring all lumps were dissolved.
5. 0.02% sodium azide (preservative) was added and the prepared chitin stored at 4^{o}C until required.

3.2.1.2 Preparation of Chitin Agar Plates

1. 400ml of single strength nutrient agar (GIBCO BRL - 9.2g/400ml) was prepared and autoclaved.
2. 100ml of double strength nutrient agar (4.6g/100ml) was prepared and autoclaved.
3. 20ml of the single strength nutrient agar was poured into each petri dish and allowed to solidify.
4. 100ml of the prepared and autoclaved 1% chitin suspension was added to 100ml of double strength nutrient agar and gently mixed.
5. The nutrient agar plates were overlaid with 10ml of nutrient agar/chitin mixture and allowed to solidify.

Preliminary growth trials with a positive chitinase producer demonstrated the ineffectiveness of this preparation to adequately reveal clear haloes in the chitin agar corresponding to areas of chitin degradation. To enhance the appearance of the haloes, the protocol for the preparation of chitin agar plates was amended by increasing the chitin concentration of the overlay from 0.5% (v/v) to 0.75% (v/v) as follows:

1. 400ml of single strength nutrient agar was prepared and autoclaved.
2. 50ml of 4x strength nutrient agar (4.6g/50ml) was prepared and autoclaved.
3. 20ml of single strength nutrient agar was poured into petri dishes and allowed to solidify.
4. 150ml of the prepared and autoclaved 1% chitin suspension was added to 50ml of 4x strength nutrient agar and gently mixed.
5. The nutrient agar plates were overlaid with 10ml of nutrient agar/chitin mixture and allowed to solidify.

3.2.1.3 Standard Chitin Agar Plates

Chitin agar plates were prepared according to the amended protocol as above. The plates were inverted and the centre of each plate was marked on the bottom. A line bisecting the plate was scribed on the bottom of each dish through the centre of the plate. The test organism was then streaked onto the agar beginning 10mm from the centre of the plate, following the scribed line, to the edge. On the opposite side of the plate, a positive control organism was streaked along the line starting from a point 10mm from the centre and following the scribed line to the edge of the plate. The control organism was isolated from chitin amended soil and identified as *Serratia liquefaciens* (Appendix B). The plates were sealed with 'Gladwrap', inverted and incubated at 25^{o}C in darkness for 72h.

3.2.1.4 Modified Chitin Agar Plates

To investigate the possibility of inherent chitinase production being triggered by a mushroom metabolite, chitin agar plates were modified to include a mushroom extract.

Chitin agar plates were prepared and a line bisecting the plate was scribed on the bottom of each dish as previously described (3.2.1.3). At the central point, a well was made in the agar with a 6mm diameter cork borer. The test organism was streaked onto the agar beginning 10mm from the central well, following the scribed line to the edge of the plate. On the opposite side of the plate, the positive control organism was streaked in the same way. The wells were filled (approximately 100μl) with a mushroom extract (see below), the plates sealed with 'Gladwrap' and incubated right way up at 25^{o}C in darkness for 72h.

Preparation of Mushroom Extract:

Three excised mushroom sporocarps were crushed in a garlic press and the fluid collected. Crushed mushroom tissue was then placed between 2 layers of fine muslin, squeezed by hand and the subsequently extracted fluid collected. The extract was centrifuged in 1ml aliquots at 14500g for

5min, then passed through a sterile 0.22μm Millipore filter membrane. The filter-sterilized extract was stored at 4^{o}C in darkness until use.

3.2.1.5 Cellophane Culture of *A. bisporus* on Chitin Agar Plates

To incorporate mushroom metabolites into chitin agar plates, *A. bisporus* was grown on cellophane discs through which the metabolites passed into the medium. Cellophane acts as a barrier to the fungal mycelium and allows for the removal of the mycelial mat easily and aseptically.
Chitin agar plates were prepared as previously described. Potato dextrose agar was used as a basal medium in place of nutrient agar, and the pH of the chitin overlay was adjusted to 5.6 to promote colonization of *A. bisporus* mycelium. Cellophane discs, 60mm in diameter, were autoclaved in distilled water, immersed in molten potato dextrose agar and laid carefully onto the chitin agar surface. Once solidified, the cellophane discs were inoculated with 6mm diameter compost malt medium agar plugs (Rainey 1989) containing *A. bisporus* mycelium, the plates sealed with 'Gladwrap' and incubated at 25^{o}C in darkness for 6 weeks. After this period, the cellophane and adherent mat of *A. bisporus* mycelium were aseptically removed. The plates were inoculated with a test organism and the positive control organism as previously described and subsequently incubated at 25^{o}C in darkness.

B] ULTRASTRUCTURAL STUDIES

3.2.2 Examination of 'Drippy Gill' Sporocarps

Excised, *Agaricus* sporocarps were inoculated with *P. agarici* and PV29 as in 2.2.4. Following incubation, tissue blocks were removed, prepared and sectioned for transmission electron microscopy (2.2.1). The interaction between the bacterium and the fungus wall was the focus of attention.

3.2.2.1 Examination of the Outer Envelope of 'Drippy Gill' Bacteria

The external 'appendages' of bacteria are often implicated in performing major roles in bacterial - host interactions. To visualize the outer bacterial membrane components, *P. agarici* and PV29 were subjected to ruthenium red staining as described by Erdos (1986) and TEM investigation.
Bacteria were incubated in NB at 25^{o}C in darkness for 48h and harvested by centrifuging 1ml of suspension in a sterile Eppendorf tube (13,500g, 10min). The resultant pellet was resuspended in a fixative solution consisting 0.3ml of 4.0% glutaraldehyde, 0.3ml of 0.2M cacodylate buffer (pH7.3) and 0.3ml of a 0.15% aqueous solution of ruthenium red and incubated at 0^{o}C for 1h. Following three buffer washes (0.2M cacodylate buffer pH7.3), the bacterial suspensions were harvested as above and treated with a secondary fixative consisting of 0.3ml of 5.0% OsO_4, 0.3ml of 0.2M cacodylate buffer (pH7.3) and 0.3ml of a 0.15% aqueous solution of ruthenium red and incubated in this solution at 0^{o}C for 1.5h. After three buffer washes, the pellet was dehydrated in an ascending ethanol series in 20% increments, infiltrated with a solution of 3 parts 100% ethanol to 1 part Epon 812 and embedded in Epon 812. After curing, 0.1μm sections were cut with a glass knife on a LKB Bromma 2128 Ultrotome and floated onto 300 mesh copper grids supported with Formvar. The grids were examined in a Jeol JEM-1200EX transmission electron microscope at an acceleration voltage of 80kV.

3.2.3 Colloidal Gold - Wheat Germ Agglutinin Labelling of Fungal Wall Chitin

A major development in carbohydrate research over the past twenty five years concerns the discovery that lectins, a group of proteins commonly of plant origin, possess the ability to selectively bind to specific carbohydrates. A lectin ("Lectin" - from the Latin *Lego* to 'pick out' or to 'choose' (Boyd and Shapleigh 1954) is simply defined as "a carbohydrate-binding protein (or glycoprotein) of nonimmune origin which agglutinates cells and/or precipitates glycoconjugates" (Goldstein *et al* 1980). This definition has been accepted by the Nomenclature Committee of the International Union of Biochemistry on the condition that the word "glycoprotein" be deleted (Dixon 1981).

Considering the important roles carbohydrates play in day-to-day intercellular processes such as adhesion, self and nonself recognition and cell signalling (Benhamou 1989), the ability to selectively isolate and label specific target carbohydrate molecules *in situ* is of great advantage.
Though their usefulness in labelling carbohydrates is undeniable, lectins remain electron transparent, thus impossible to detect. As a consequence, the lectin must be tagged with an electron dense particulate marker such as ferritin, haemocyanin, iron-dextran or iron-mannan (Benhamou 1989).
More recently, colloidal gold, which has the following properties, has become an attractive alternative. It is non cytotoxic, stable, composed of particles of uniform size, easily prepared, electron dense and applicable to many methods of microscopy.
Originally used to study intracellular exchanges in the amoeba *Chaos chaos* (Feldherr and Marshall 1962), applications of colloidal gold as an electron opaque marker have widened to include transmission electron microscopy as initially described by Faulk and Taylor (1971), scanning electron microscopy (Horisberger *et al* 1975) and fluorescence microscopy (Horisberger and Vonlanthen 1979).

Colloidal gold is a negatively charged hydrophobic sol whose stability is maintained by electrostatic repulsion (Goodman *et al* 1981) and is produced by the reduction of tetrachloroauric acid ($HAuCl_4$) by sodium

citrate (Frens 1973). The size of particle produced is determined by altering the volume and concentration of the reacting reagents. As a result of adsorbed ions in the sol, the gold particles carry a net negative charge. Subsequent adsorption of macromolecules onto the particle surface results in the colloid becoming hydrophilic (Horisberger 1989).

Lectins predominantly interact with the non-reducing terminal glycosyl groups of polysaccharides and glycoproteins. Wheat germ agglutinin, an exception to this general rule, has the ability, as well as interacting with the glycosyl groups, to interact with internal N-acetylglucosamine residues (Allen *et al* 1973). Unlike the majority of lectins, wheat germ agglutinin is not a glycoprotein, as demonstrated by the absence of carbohydrate found during the purification and analysis of wheat germ agglutinin by Allen *et al* (1973), a finding contradictory to that of Nagata and Burger (1974).

Wheat germ agglutinin, derived from *Triticum vulgaris*, is highly specific for N-acetylglucosamine and possesses a binding site which is able to bind a sequence of three ß-(1,4)-linked residues of this sugar (Greven and Peters 1986) and consequently has a high affinity for oligomers and polymers of N-acetylglucosamine, particularly chitin (Allen *et al* 1973, Nagata and Burger 1974, Goldstein *et al* 1975, Sharon and Lis 1972).

The lectin-colloidal gold conjugate is prepared by the noncovalent electrostatic adsorption of the protein to the gold particle which stabilizes the sol to flocculation (Benhamou 1989). The adsorption of proteins on gold sols is irreversible (Horisberger *et al* 1975). The conjugation, however, is very tenuous, the adsorption process being dependent upon several physicochemical parameters. Among these in particular are protein concentration, interfacial tensions, molecular electrostatic charges and the pH condition of the solution and its correlation with the isoelectric point (the pH of solution at which the net charge on the molecule is zero) of the protein (Geoghegan and Ackerman 1977, Goodman *et al* 1981). Failure to maintain optimal conditions throughout the reaction results in poor complex formation and unstable solutions. With the recognition of the influence that the outlined physicochemical factors have on conjugate quality and yield, the preparation of lectin-gold complexes has become simple and easily reproducible (Benhamou 1989).

Despite taking into consideration the physicochemical factors cited above, it was found that wheat germ agglutinin was unable to successfully stabilize gold sols due to the low molecular weight of the lectin (Benhamou and Ouellette 1986, Roth and Binder 1978, Horisberger and Rosset 1977). Particles labelled with proteins of such low molecular weights as wheat germ agglutinin (MW 36,000) were found to flocculate slowly in buffers (Horisberger and Rosset 1977). Both gold particle size (Roth and Binder 1978) and the high isoelectric point of wheat germ agglutinin (7.9-9.4) have also been suggested as influencing adsorption of the lectin (Geoghegan and Ackerman 1977). To complex wheat germ agglutinin successfully with colloidal gold, the molecular weight of wheat germ agglutinin may be increased by cross-linking with bovine serum albumin using a gluteraldehyde bridge (Horisberger and Rosset 1977).

An alternative method of counteracting the consequences of stabilizing a colloidal gold sol with a protein of low molecular weight, involves introducing an intermediate protein of sufficiently high molecular weight to successfully bind to and stabilize the gold sol. The preferred intermediate protein must possess the ability to be adsorbed successfully onto gold particles thus stabilizing the sol, and also the ability to bind specifically to wheat germ agglutinin (Fig. 3.1). Ovomucoid, a water soluble glycoprotein with an isoelectric point of between 3.9-4.3 (Geoghegan and Ackerman 1977) has been selected due to its affinity for wheat germ agglutinin, a property demonstrated by LeVine *et al* (1972), Geoghegan and Ackerman (1977) and Roth (1983). After adjusting the gold sol to the appropriate pH (5.2-5.5), an aqueous solution of ovomucoid is used to stabilize the colloidal gold. The ovomucoid-gold conjugate is then recovered by centrifugation and resuspending the resultant pellet in phosphate-buffered saline containing polyethylene glycol (MW 20,000), which assists in the stabilization of the conjugate by preventing aggregation of gold particles. This method of stabilizing colloidal dispersions (so-called "steric stabilization") was investigated as far back as the mid-nineteenth century by Faraday, who used gelatin, amongst other macromolecules, to prevent colloidal gold from coagulating in the presence of ions. It is thought the adsorption of polymers on to the particle surface results in long, flexible polymer chains protruding out into the solution thus keeping particles at a sufficient distance to prevent interparticulate coherence via Van der Waals forces (Horisberger 1989).

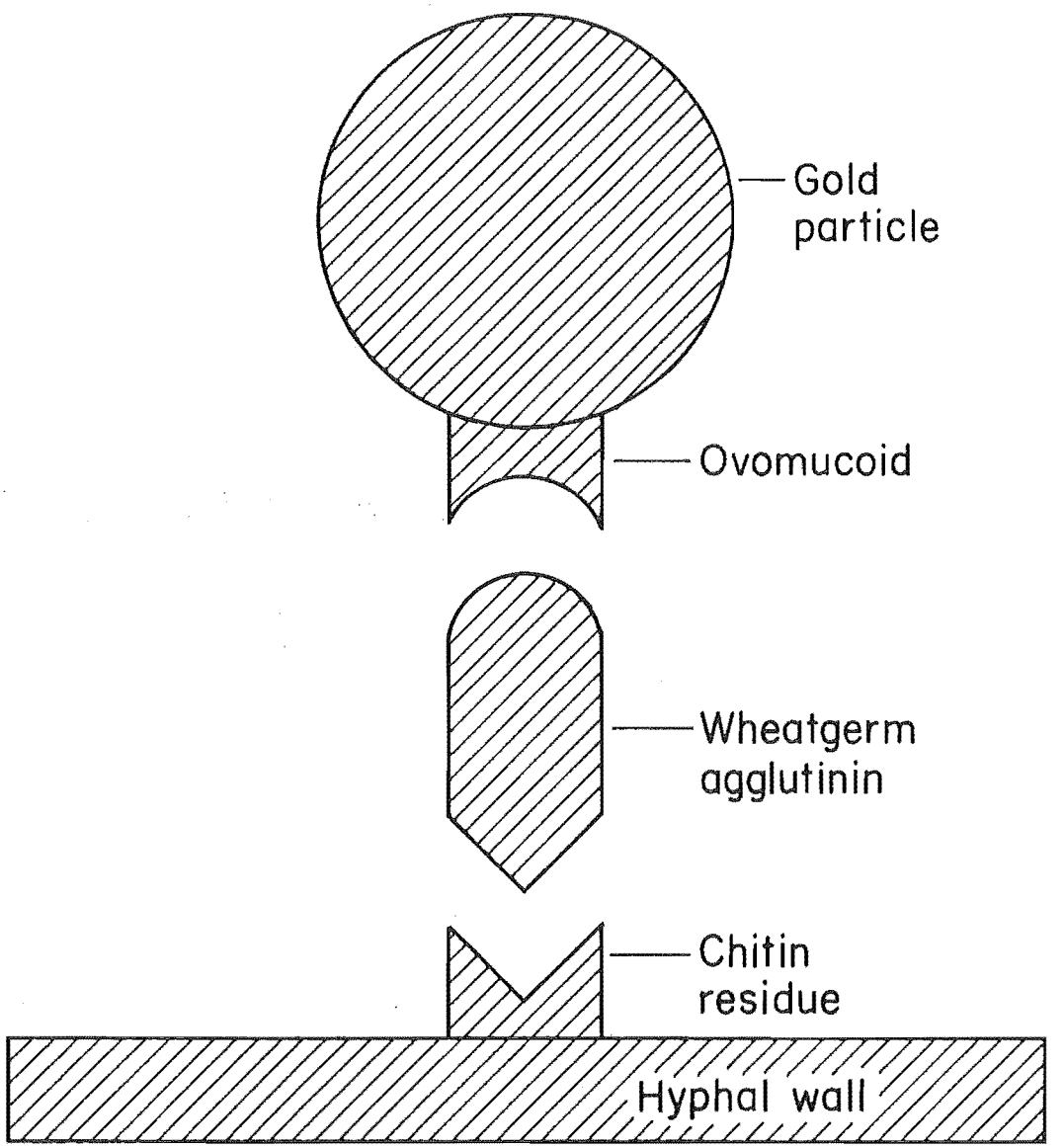


Figure 3.1 The colloidal gold-Ovomucoid-wheat germ agglutinin labelling system.

To locate chitin in the sample being investigated, resin-embedded thin sections are incubated with unlabelled wheat germ agglutinin which binds specifically to N-acetylglucosamine. The consequent sugar-lectin complex is then detected by treatment with the colloidal gold-ovomucoid conjugate. Ovomucoid, chosen for its high affinity for wheat germ agglutinin, binds to the sugar-bound lectin, thus visualization of N-acetylglucosamine *in situ* is achieved (Benhamou 1989).

In order to realize optimal binding of wheat germ agglutinin to N-acetylglucosamine and subsequent binding of the ovomucoid-gold conjugate to the sugar-bound lectin, various factors must be taken into consideration whilst preparing the specimen.
Initially, gold labelling of N-acetylglucosamine was attempted on tissue samples prepared for transmission electron microscopic examination of diseased sporocarps as previously described (2.2.1). Failure to visualize gold particles on tissue prepared in such a way (embedded in Spurr's resin and mounted on copper grids), was attributed to two major faults. Firstly, Spurr's resin is largely impermeable to cytochemicals and is therefore not suitable for the post-embedding staining technique (Manfred Ingerfeld, Department of Plant and Microbial Sciences, University of Canterbury, pers. comm.). Secondly, copper grids are not recommended for use with cytochemicals as they tend to oxidize readily during incubation with the gold complex and contaminate the sections. Grids of relatively inert metals, such as nickel and gold, are therefore advised (Benhamou 1989).

LR White (London Resin Company Limited), a hydrophilic methacrylate resin, was used to embed specimens for the cytochemical examination of 'drippy gill' diseased sporocarps. This embedding agent allows the passage of aqueous solutions of, or near, neutral pH without the need for pre-treatments such as hydrogen peroxide etching or protease digestion. Because N-acetylglucosamine, in its polymerized form, chitin, is a structural component of the fungal cell wall, it was considered unlikely to alter when subjected to various cytochemical treatments. Consequently, many of the recommendations published in London Resin's applications leaflet, designed to preserve and enhance antigenicity, were disregarded. Indeed, implementing some of these recommendations resulted in a poor labelling intensity of gold over the fungal cell wall, largely due to inadequate

membrane preservation. Through a process of trial and elimination, a protocol was developed utilizing aspects from both London Resin's applications leaflet and standard TEM specimen preparation methodology, which yielded an acceptable labelling density over fungal cell walls.

3.2.3.1 Glassware Preparation.

Throughout the process of producing a colloidal gold sol, it is extremely important to ensure that the cleanliness of all glassware and plasticware the sol is to come into contact with is scrupulously maintained. Any particulate matter adhering to the sides of a vessel may cause a cloudiness in the gold sol or promote an undesirable aggregation of gold particles. Failure to chemically treat the glassware correctly also results in the formation of a gold mirror on the sides of the vessel, depleting the eventual yield of gold conjugate. To minimize the risk of contamination and mirror formation, all glassware and plasticware were first autoclaved (121^{o}C, 20min) then washed twice with Pyroneg (Diversey New Zealand Limited) in hot water, rinsed twice in hot water, then three times in double-distilled water, followed by autoclaving a second time. Once cool, the glassware and plasticware were rinsed in a solution of 5% dimethyldichlorosilane (BDH) in chloroform, left in a fume hood for 2h to allow the chloroform to evaporate, depositing dimethyldichlorosilane on the glass, then baked for 2h at 180^{o}C. Before use, the glassware and plasticware were rinsed several times in double-distilled water.

3.2.3.2 Preparation of the Colloidal Gold Sol.

A colloidal gold sol consisting of particles averaging 14nm in diameter was prepared according to Frens (1973) as described by Benhamou (1989).

1. To 95ml of double-distilled water, 5ml of 0.2% tetrachloroauric acid (Sigma) aqueous solution were added and the solution allowed to boil for 5min.
2. As quickly as possible, 4ml of 1% sodium citrate were added and the solution allowed to boil gently. The solution turned blue - purple - dark red

and finally orange-red. The reduction was complete after 10-15min of boiling.

3.2.3.3 Preparation of the Ovomucoid-Colloidal Gold Conjugate.

An indirect labelling technique using Ovomucoid as an intermediary molecule between the gold and wheat germ agglutinin was selected because of the difficulties encountered in conjugating a molecule of such low molecular weight as wheat germ agglutinin to metal particles.

1. The colloidal gold sol was adjusted to pH5.2-5.5 with 0.1N HCl.
2. 20μg of Ovomucoid (Sigma) were dissolved in 0.1ml double-distilled water and used to stabilize 10ml colloidal gold.
3. The complex was then centrifuged (60min, 20,000g, 4^{o}C) and the resultant dark red pellet resuspended in 0.5ml of 0.1M PBS-PEG, pH6.0.

The Ovomucoid-gold conjugate remains active for many months stored at 4^{o}C.

3.2.3.4 Sample Preparation.

1. Excised sporocarps of *A. bisporus* were inoculated with *P. agarici* isolates PMS601, PMS752 and PV29 as previously described and left in a darkened humid chamber at room temperature to develop 'drippy gill' symptoms.
2. After 4-5d, tissue sections approximately 3mm x 2mm were cut from four locations (outer stipe, hymenium, cap interior and top of cap) and fixed in 1% gluteraldehyde in 0.1M PO_4 buffer pH7.3 for 3h.
3. Following a short buffer wash (0.1M PO_4 buffer pH7.3), the tissue blocks were transferred to 1% OsO_4 in 0.075M PO_4 buffer pH7.3 for 3h.
4. Tissue blocks were then washed three times in buffer (0.1M PO_4 pH7.3) and left overnight in buffer at 4^{o}C.
5. Blocks were dehydrated through a graded ethanol series (20-100% in 20% increments), 30min in each concentration, followed by two further changes of 100% ethanol, each for 30min.
6. Tissue blocks were then immersed in diluted LR White (2:1 LR White:100% ethanol) and left at room temperature for 1h.

7. This was followed by a change to 100% LR White for 1h.
8. 100% LR White was changed again and tissue blocks were left overnight on a rotamix at room temperature.
9. Following a further change of 100% LR White for 1h, the tissue blocks were transferred to pre-dried gelatin capsules (50^{o}C for 4h) which were filled with 100% LR White and polymerized at 50^{o}C for 24-36h.
10. After polymerization, mesas were cut by hand with a razor blade and sections 0.1μm thick (cut with a glass knife on a LKB Bromma 2128 Ultrotome) were floated onto both supported (Formvar) and non-supported 300 mesh gold grids.

3.2.3.5 Indirect Localization of N-Acetylglucosamine.

The following protocol is based largely on that described by Benhamou (1989).

1. Thin sections of embedded tissue mounted on gold grids were floated face down on a drop of 0.01M PBS (pH7.2) for 10min in a moist chamber.
2. Grids were transferred to a drop of wheat germ agglutinin (25μg/ml 0.01M PBS pH7.2) for 30min at room temperature.
3. Sections were then jet washed with 0.01M PBS pH7.2, immersed in PBS for 5min and jet washed again to remove any unbound lectin from the surface of the section. Grids were blotted dry by touching their edges with a filter paper, being careful not to allow complete drying of the sections.
4. Grids were incubated on a drop of the Ovomucoid-gold complex diluted 1:30 in 0.1M PBS-PEG (pH6.0) for 30min at room temperature.
5. Grids were then washed thoroughly with PBS, rinsed in double-distilled water and air dried.
6. Finally, the sections were counterstained with uranyl acetate (1% solution in 50% ethanol) and lead citrate (Sato 1967).
7. Sections were then examined in a Jeol JEM 1200 transmission electron microscope at an acceleration voltage of 80kV.

3.2.3.6 Controls and Specificity Trials

To ensure the specificity of both sugar-lectin binding and the affinity of the Ovomucoid-gold conjugate for the sugar-bound lectin, two controls were performed.

1. Thin sections on gold grids were firstly floated face down in a drop of 0.01M PBS (pH7.2) for 10min then incubated with Ovomucoid-gold complex for 30min at room temperature. The grids were rinsed carefully in PBS and double-distilled water. Lack of visible gold on the specimen demonstrates specificity of the Ovomucoid-gold to the lectin and its inability to bind directly to N-acetylglucosamine.

2. Thin sections on gold grids were floated face down on a drop of 0.01M PBS (pH7.2) for 10min, then incubated in wheat germ agglutinin (25μg/ml 0.01M PBS pH7.2) at room temperature for 30min, followed by a jet wash with 0.01M PBS (pH7.2), a rinse in PBS for 5min then another jet wash in PBS. Grids were then transferred to Ovomucoid (200μg/ml 0.01M PBS pH7.2) for 30min at room temperature, and were finally incubated in Ovomucoid-gold complex for 30min at room temperature. The grids were jet washed with PBS, then briefly rinsed in PBS followed by double-distilled water. In the first instance, the lectin binds to the sugar. Subsequent incubation with Ovomucoid 'satisfies' all the available binding sites on the sugar-bound lectin. Incubation with Ovomucoid-gold complex therefore results in no gold attachment to the section as the lectin-Ovomucoid binding has previously been completed.

Tests were completed to demonstrate, in small part, the specificity of the labelling system.

1. Thin sections of *Pinus radiata* roots embedded in LR White demonstrating a mycorrhizal association with *Rhizopogon rubescens* (Williamson 1993) were obtained (Wendy Williamson, Department of Plant and Microbial Sciences, University of Canterbury, pers. comm.) and labelled according to the schedule (3.2.3.5). For examination, areas of sections were selected which had a root cell wall closely adjacent to a

fungal cell wall. Gold label is expected to be located specifically attached to the chitinous fungal wall demonstrating preferential binding of the gold particles to one glucose polymer (chitin) over another, cellulose, (an α-(1,4) glucan), which is the major skeletal component of plant cell walls.

2. Mycelium of *Phytophthora nicotianae* (ICMP7753), an Oomycete which possesses cellulose as a skeletal polysaccharide instead of chitin, was fixed, embedded and labelled as per schedule (3.2.3.4 and 3.2.3.5). In this case, the walls are expected to be entirely devoid of gold particles, demonstrating that the specific preferred substrate for binding in fungal cell walls is chitin.
The procedure was repeated on mycelium of *Saprolegnia ferax* (ATCC36051), an Oomycete which does possess chitin as a major skeletal polysaccharide. Consequently, hyphal walls are expected to be labelled with gold particles in contrast with the situation visualized with *P. nicotianae*.

3.2.3.7 Analysis of Gold Labelling

Gold particles adhere preferentially to chitin molecules via the highly specific interaction of two proteins, wheat germ agglutinin and Ovomucoid (3.2.3). Assuming the presence of gold on hyphal walls is a direct indication of the presence of chitin, then the density of particulate gold may be indicative of the amount of chitin present in the wall. Hence, if the chitin content of the wall was to be reduced, this would be reflected in a parallel reduction of the density of wall-bound gold particles. From this, the comparative amount of chitin in hyphal walls may be assessed.

Initially, a test was carried out to determine the efficacy and reproducibility of this method. Because the chitin content of Basidiomycete hyphal walls is known to change with age and tissue location/function (Garcia Mendoza *et al* 1987), two tissue blocks were removed from the outer stipe of one 'clean' excised *Agaricus* sporocarp. The two tissue blocks were fixed, embedded, sectioned and floated onto separate 300 mesh unsupported gold grids as previously described (3.2.3.4). Both grids were subjected to the protocol for the indirect localization of chitin (3.2.3.5) using the same reagents and solutions, though each was treated in a separate staining

well. Once counter stained, each grid was viewed as before at 80kV and micrographs of gold labelled walls were taken at 75,000x magnification. Once the micrographs had been enlarged, the wall lengths were divided into $100nm^2$ units and the number of gold particles per unit area were counted and noted. Once all counts were collated for both grids, standard ANOVA techniques were applied (Sokal and Rohlf 1981) to analyse the significance of variance which may exist within and between the two samples.

Following this control, further analyses were carried out to determine whether the presence of bacteria impose a significant variance on the density of gold label, due to the degradation and consequent reduction of hyphal wall chitin.

Tissue from the stipe of an excised *Agaricus* sporocarp exhibiting classic 'drippy gill' symptoms was prepared, the hyphal wall chitin labelled with gold and observed as described above. Wall lengths with bacteria present (within 250nm) and wall lengths with bacteria absent (extracellular matrix intact and continuous with the hyphal wall), were selected from the same grid and photgraphed. The density of particulate gold was measured as before and compared using a nested ANOVA technique (Sokal and Rohlf 1981) to demonstrate if any influence which may be exerted by the bacteria on the density of wall-bound gold and consequently hyphal wall chitin, is significant.

3.2.4 Detection of Glucan Degradation

Two tests were employed in an effort to detect degradation of the polysaccharide (glucan) fractions of *Agaricus* walls in response to enzymatic attack by *P. agarici* and PV29.

3.2.4.1 Thiery's Test for Polysaccharides

Thiery's test (TCH or thiocarbohydrazide test) for polysaccharides (Thiery 1967), enables polysaccharides to be visualized by transmission electron microscopy.

This reaction is dependent upon the detection of aldehyde groups generated by the oxidation of free hydroxyl and/or amino groups on adjacent carbon atoms of the sugar being investigated. These periodate-induced aldehydes condense with hydrazine groups, which in turn reduce silver proteinate, resulting in deposition of fine particles of electron-dense metallic silver at the site of the reaction (Erdos 1986).

The reaction is dependent upon the presence of aldehyde groups, therefore, the use of glutaraldehyde as a fixative and osmium tetroxide (which introduces reactive keto groups) in tissue processing is discouraged to avoid false positive polysaccharide staining (Erdos 1986) and comprehensive controls are recommended (Courtoy and Simar 1974). However, the composition of the *Agaricus* wall, as a whole, has been shown to be comprised almost exclusively of polysaccharide with the exception of a few minor components such as melanins, proteins and lipids (3.1.2). The portion of *Agaricus* wall being investigated by this technique is composed entirely of polysaccharide (predominantly α-(1-3) and ß-(1-3)-glucans) and therefore any discrepancy visualized in wall structure will be due to an alteration in the state of the glucan. For this reason, it was considered unnecessary to heed the precautions, as this technique is being used to delineate glucan layers rather than localizing glucan amongst a host of other compounds.

The following protocol was used on tissue samples prepared for the transmission electron microscopic examination of diseased sporocarps as previously described in 3.2.3.4. Thin sections were floated on to non-coated 300 mesh gold grids.

1. Grids were placed in 1% periodic acid for 30min in a porcelain spotting plate and immersed to ensure thorough wetting.
2. Following two brief washes in distilled water, the grids were further washed in two longer changes of distilled water, each for 10min duration.

3. The grids were then thoroughly immersed in 0.2% thiocarbohydrazide in 20% glacial acetic acid and left for 24h.
4. They were passed through a decreasing glacial acetic acid concentration series: 20%, 15% then three changes of 10%. Each step was left for 20min.
5. The grids were placed briefly in 5% glacial acetic acid, followed by 2% and then distilled water, with three further changes of distilled water for a period of 20min each.
6. A 1% silver proteinate solution was made up in distilled water in darkness and the grids were immersed in this solution for 30min in complete darkness.
7. Grids were rinsed briefly in three changes of distilled water, dried by touching the grid with the edge of a filter paper and stored.
8. The grids were examined in a Jeol JEM-1200EX transmission electron microscope at an acceleration voltage of 80kV within 7 days of staining, to avoid deterioration.

3.2.4.2 Colorimetric Assays

Preliminary investigations

Three glucose polymers, laminarin, a ß-(1,3) glucan, nigeran, an α-(1,3) glucan and pullulan, a ß-(1,3)-(1,6) glucan were used as substrates to test the production of glucanases from the 'drippy gill' organism *P. agarici*, PV29 and a known ß-glucanase producer, PMS164 (*P. gladioli* p.v. *agaricicola* - Appendix A)

The degradation of the test substrates was indicated by the presence of glucose, the basic compound unit of glucan polymers and the end product of total glucan hydrolysis. The assay solution of Reese and Mandels (1959) was utilized and glucose was detected with Clinistix (Bayer Diagnostics), a glucose oxidase-dye system presented as a solid reagent, which reacts exclusively with glucose. Approximate concentrations (>14mmol) of glucose are indicated by the intensity of the colour change of the incorporated dye.

Each bacterial isolate was tested on all three glucan substrates for the possibility of a constitutive hydrolysing enzyme and an enzyme induced by the presence of a particular glucan.

Constitutive Enzyme Assay

Assay solution: 0.6% glucan (laminarin and pullulan) in 0.05M citrate buffer pH4.8. The insoluble glucan, nigeran, was used as above, but as a suspension compared with the solutions of the readily soluble glucans.

Enzyme solution: 10ml of 24h shaken NB cultures of *P. agarici*, PV29 and PMS164 (30^{o}C, 200rpm in darkness) were centrifuged (11,000g, 4^{o}C, 10min) and the resultant supernatant was filter sterilized through a 0.22μm Millipore membrane into sterile universal bottles. The subsequent cell-free culture filtrates were stored at 4^{o}C in darkness until required. As a control, uninoculated NB containing 0.3% glucan was treated identically to the cell-free culture filtrates.

Test procedure: 0.5ml of assay solution was aseptically added to 0.5ml of enzyme solution in a sterile 1.5ml Eppendorf tube and incubated for 1h at 40^{o}C in darkness, after which 10μl were aseptically removed and applied to a Clinistix reagent strip. The change of colour intensity was recorded within 5min of application. The Eppendorfs were incubated for a further 23h at 40^{o}C in darkness, after which time the solution was retested and any colour change again noted.

In an effort to parallel ambient conditions in a mushroom culture bed, trials were carried out using an assay solution of pH6.4, the pH of mushroom macerate (50g mushroom blended at high speed for 10min in 150ml distilled water). A different incubation temperature was also trialled, again to approximate the conditions experienced on a commercial mushroom bed. The results of these trials, using soluble laminarin as the substrate and the positive ß-glucanase producing control organism, indicated that incubation at 40^{o}C and an assay solution pH of 4.8 were more conducive to optimal enzyme activity than the alternative combinations of temperature and pH. Consequently, all subsequent assays were carried out under these conditions.

Inducible Enzyme Assay

Assay solution: 0.6% glucan (laminarin and pullulan) in 0.05M citrate buffer pH4.8. As before, the insoluble nigeran was added as a suspension rather than solution.

Enzyme solution: NB containing 0.3% glucan (to parallel the final concentration of the glucan in the assay solution) was inoculated with the test organisms and incubated in darkness in an orbital shaker ($30^{o}C$, 200rpm) for 24h. The cultures were centrifuged and filtered as before to yield cell-free culture filtrates. These were again stored at $4^{o}C$ in darkness until required. A control consisting of uninoculated NB containing 0.3% glucan was again used for comparison.

Test procedure: Initially, the cell-free culture filtrates were tested directly with Clinistix to detect glucose produced in culture. Following this, the enzyme solutions were tested as before with the assay solution of Reese and Mandels (1959).

HBH Method for the Determination of Reducing Sugars

Clinistix react exclusively with glucose, the ultimate product of glucan degradation. Glucans, however, are not necessarily hydrolysed directly to glucose, a number of intermediate reducing sugars having been identified, such as laminaribiose (Reese and Mandels 1959) and laminaritriose and gentibiose (Bull and Chesters 1966). The products of glucan hydrolysis are also dependent upon the mode of action of the hydrolysing enzyme. ß-D-(1,3) glucanases are of two types. They are the endoglucanases, a random splitting type, which hydrolyse ß-(1,3)-glucans (for example) to laminaribiose and other oligosaccharides and secondly, the exoglucanases, the endwise splitting type, which yield glucose as the sole product (Reese and Mandels 1959). In an effort to detect the alternative reducing sugars yielded from glucan hydrolysis and low concentrations of glucose (<14mM), the HBH (4-hydroxybenzoyl hydrazine) method reported by Lever (1973) was used, as described by Jarvis (1992).

HBH Reagent: 1.84g tri-sodium citrate and 0.368g calcium chloride were dissolved in a small amount of distilled water. 75ml of 1M NaOH and 1.9g of 4-hydroxybenzoyl hydrazine were added and made up to 250ml with distilled water. The solution was stored at 4^{O}C in darkness until required, it being stable for approximately four weeks.

Test solution: The test organisms, *P. agarici*, PV29 and PMS164 were each inoculated into three tubes of NB each containing one of the following: 0.3% laminarin, 0.3% nigeran and 0.3% pullulan and were incubated in shaking culture for 24h in darkness (30^{O}C, 200rpm). These three glucans were selected for further in depth assay as their existence in fungal walls and/or extracellular mucilage has been established (3.1.2). The cultures were centrifuged (11,000g, 4^{O}C, 10min) and the supernatant passed through a 0.22μm Millipore filter into sterile universals. The resultant cell-free culture filtrate was stored in darkness at 4^{O}C until required. The control treatment of 0.3% glucan in NB was treated identically to the cell-free culture filtrates.

Procedure: Each test solution (40μl), including a NB blank, was mixed with 5ml of HBH reagent in a glass test tube and covered with a foil cap. The tubes were heated in a boiling water bath for 5min after which time the tubes were immediately cooled in cold water and the absorbance read at 420nm in an Hitachi U-2000 spectrophotometer. Each test solution was treated in triplicate. The sugar concentrations of the test solutions were calculated from a standard glucose concentration curve which was created using glucose concentrations ranging from 0mM to 20mM, dissolved in NB. Glucose was selected as it is the ultimate end product of complete glucan hydrolysis.

Because of the light sensitive nature of the HBH reagent and in particular the coloured end product, exposure to bright light during the procedure was avoided.

3.2.5 Isolation and Digestion of *Agaricus* Wall Fractions

Crude preparations were made of the three major *Agaricus* wall fractions and each was subjected to the action of 'drippy gill' isolates and PMS164, a known ß-glucanase producer. Glucan degradation was detected by determining the presence of glucose in the test solution utilizing Clinistix.
The water soluble extracellular mucilage was extracted by a modified method of both Avellan *et al* (1986) and Wessels and Niederpruem (1967).
1. 50g of *Agaricus* sporocarp were blended in 100ml of hot distilled water ($60^{o}C$) at high speed for 15min and the suspension was maintained for 40h at $50^{o}C$.
2. The suspension was then centrifuged (10min, $20^{o}C$, 13,000g) and washed six times to remove the viscous water soluble polysaccharide. The supernatants and washings were kept and concentrated to approximately one tenth of their original volume.
3. S-glucan was extracted from the remaining pellet by treating it with 1N KOH for 18h at $25^{o}C$ and precipitating out the glucan by adjusting the pH to 5.0 with acetic acid.
The remaining KOH insoluble material was considered to be R-glucan. The glucan extracts were dried and ball-milled to a fine powder.

The water soluble polysaccharide was sterilized ($121^{o}C$, 15min), 2ml aliquots inoculated with the test organism and incubated at $30^{o}C$, 200rpm in an orbital shaker in darkness for 24h. The powdered glucans were added to 5ml NB to create a 0.3% suspension, sterilized, inoculated with the test organisms and incubated as above. Controls to test the dissociation of the extracts were included for all treatments. Glucose evolution was tested initially on the crude inoculum with Clinistix. Subsequently, the cultures were centrifuged (11,000g, $4^{o}C$, 10min) and the resultant supernatant was filter sterilized through a 0.22μm Millipore filter membrane into sterile universal bottles. 0.5ml of the assay solution of Reese and Mandels (1959) containing laminarin (ß-(1,3)-glucan being the more prevalent in *Agaricus* walls) was added to 0.5ml of each of the sterile cell-free culture filtrates and incubated at $40^{o}C$ for 24h. After this period, the test solutions were tested for the presence of glucanase (indicated by the evolution of glucose) with Clinistix reagent sticks.

3.2.6 The Enzymatic Degradation of *Agaricus* Mycelium

To visualize the effects enzyme degradation has on *Agaricus* tissue, vegetative mycelium was subjected to ß-glucanase, chitinase and a combination of both enzymes over a period of days. Mycelium from the growing margin of a 10d culture on CMM was scraped from the agar surface with a sterile scalpel blade and immersed in chitinase (1mg/ml distilled water) in a sterile 1.5ml Eppendorf tube. Two further scrapings were placed into two separate Eppendorfs containing B-glucanase (1mg/ml distilled water). All three tubes were incubated at 25^{O}C in darkness for 4d. Following incubation, one sample from a ß-glucanase tube was transferred to an Eppendorf containing chitinase and reincubated for 4d. The mycelia were removed from each tube, rinsed in distilled water and prepared and sectioned for TEM investigation (3.2.3.4). Each sample was subjected to both the gold labelling protocol for the indirect localization of N-acetylglucosamine (3.2.3.5) and Thiery's test for polysaccharides (3.2.4.1). The effect of each treatment was visualized utilizing the TEM. The effects the pure enzyme treatments had on *Agaricus* mycelium were compared with tissue from 'drippy gill' affected sporocarps and comparisons noted in an effort to explain the influence exhibited by the bacteria on the hyphal walls.

3.2.7 The Adhesion of *P. agarici* and PV29 to *Agaricus* Mycelium

The procedure of Rainey (1989), modified from Preece and Wong (1982), was closely followed to evaluate the ability of *P. agarici* and PV29 to adhere to mushroom mycelium. The results generated by Rainey (1989), demonstrate the effect that different culture conditions impose on the ability of a pathogenic pseudomonad, *P. tolaasii*, to adhere to host mycelium. However, as both *P. agarici* and PV29 are capable of inducing 'drippy gill' symptoms on excised *Agaricus* sporocarps after inoculation directly from

KB agar plates, this medium was considered sufficient to culture the bacteria.

Preparation of bacterial suspensions: *P. agarici* and PV29 were cultured overnight at 25°C on KB agar and removed by inoculating loop and suspended in sterile distilled water to give an A_{610} of 0.4 (approximately 4 x 10^8 cells/ml). The suspension was vortex mixed for 1min and examined microscopically to ensure cells were motile and not clumped together. The suspension was repeatedly mixed until homogeneous.

Determination of bacterial numbers: The method of Miles and Misra (1938) was employed to estimate concentrations of viable bacterial cells in solution. 20μl aliquots of bacterial suspension were dropped onto fresh nutrient agar plates supplemented with 0.3% yeast extract. Dilutions (0 to 10^{-5}) were made in PBS in sterile 1.5ml Eppendorf tubes and all equipment contacting the suspensions was made from poly-propylene, previously demonstrated not to support adherence of bacterial cells (Rainey 1989).

Preparation of Agaricus mycelium: 10d cultures of *A. bisporus* growing on CMM (25°C) were flooded with sterile distilled water for 15min to suppress aerial mycelium. Agar plugs were removed from the periphery of the colony with a sterile 10mm diameter cork borer and transferred to sterile petri dishes and left to dry for 15min.

Attachment assay: Once the mycelial plugs had dried, a 20μl aliquot of bacterial suspension was carefully applied to each plug and gently spread over the entire surface with the pipette tip ensuring the tip did not come into contact with the mycelium and that no bacterial suspension was lost over the side of the plug. The number of bacteria within the original suspension was calculated immediately following inoculation of the mycelial plugs.
The inoculated plugs were incubated for 30min at 25°C in darkness, after which time they were immersed in 10ml sterile PBS in universals and vortex mixed for 1min to dislodge any bacteria not adhering firmly to the mycelium. By counting the concentration of bacteria in the 10ml PBS and subtracting this value from the concentration of the initial suspension, the number of bacteria adhering to the hyphal surfaces, expressed as a percentage, was estimated. The assay was replicated five times for each bacterium.

3.3 RESULTS

3.3.1 Petri Dish Assay

The modification to the original protocol for the production of standard chitin agar plates provided a medium which enhanced the visible halo surrounding the positive control, *Serratia liquefaciens*. The visible halo corresponded to an area devoid of chitin, it having been hydrolysed by the bacterium. The lack of similar visible haloes around the test organisms *P. agarici* and PV29 suggests these bacteria are unable to hydrolyse chitin suspended in the medium (Fig. 3.2). Modifications to the chitin agar plates, designed to incorporate *Agaricus* metabolites into the medium, yielded similar results. Mushroom extract, consisting of macerated tissue, delivers to the medium a host of cytoplasmic and wall constituents which the bacteria would encounter during pathogenesis, particularly if the bacteria were intrahyphal. Similarly, the cellophane culture of *Agaricus* provides the medium with a range of extracellular products and secondary metabolites which the bacteria would again encounter during pathogenesis and in the mushroom hyphosphere. These two methods, therefore, subject the bacteria to a wide range of possible triggers inducing latent chitinase activity. However, the results mirror those for standard chitin agar plates. The positive control organism clearly degrades chitin, evident as a halo of clearing, while the two 'drippy gill' test bacteria do not apparently clear the plate (Fig 3.3) and are therefore regarded as negative in terms of chitinase production.

3.3.2 T.E.M. Ultrastructure

Initial investigations with both PV29 and *P.agarici* isolates demonstrated that the effects these bacteria have on *Agaricus* tissues are identical. Similarly, these effects are independent of tissue type. The amount of bacteria present in section is, however, somewhat increased in stipe tissue,

in which the individual hyphae are less densely packed compared with other tissues and consequently, the interhyphal spaces are more extensive.

The great majority of bacteria were found to be extrahyphal, though there was evidence of intrahyphal subsistence. Those hyphae which contain bacteria are, compared with adjacent 'unoccupied' hyphae, devoid of the organelles and ground cytoplasm associated with healthy fungal cells (Fig. 3.4a). The presence of extracellular bacteria is associated with a degradation of the hyphal extracellular matrix (Fig. 3.4b), which appears as an intertwining network of very fine microfibrils when undisturbed. Degradation of the extracellular matrix requires the immediate presence of the bacterium. Even when not present in section, the presence of the 'drippy gill' bacterium is indicated by the appearance of larger microfibrils, presumably originating from the bacterium. These large microfibrils are seen in association with areas of clearing within the extracellular matrix (Fig. 3.4c), confirming the ability of these bacteria to degrade such material.

Bacteria in close proximity to the hyphal wall apparently disrupt the outer glucan layers, giving them an almost 'woolly' appearance (Fig. 3.4b), while others have a seemingly more detrimental effect on hyphal walls. These effects range from a reduction in wall integrity (Fig. 3.4d) through to an apparent restricted penetration of the wall (Figs. 3.5a, 3.5b, 3.5c, 3.5d and Fig. 3.6a). The influence of the bacteria on the fungal wall is manifest by a reduction of wall integrity, shown as a lateral diffusion of wall components and a disruption of the outer polysaccharide (Fig. 3.4d). The extracellular matrix surrounding individual bacteria has been removed, although some areas remote from the bacteria retain their intact matrix. As before, there is evidence supporting the intrahyphal existence of the bacterium (Fig. 3.4d) and as in Fig. 3.4a, the occupied hyphal cell is devoid of cellular material. Often, penetration of fungal walls is accompanied by a disruption to the underlying plasma membrane (Figs. 3.5a, 3.5b) which is characterized by a loss of integrity and dispersal of material (Fig. 3.5c). Hyphal cells lacking living components appear to be a prerequisite for invasion as seen in Fig. 3.5d, where both of the hyphal cells containing bacteria have little or no cellular organization, compared with adjacent healthy cells. As before, the

extracellular matrix remains intact in pockets which are removed from the influence of the bacteria. Looking closely at the site of penetration (Fig. 3.6a), the exposed broken abutments appear ragged as though forcibly parted, as opposed to the diffuse 'eroding' which would be expected from an enzymatic degradation. Caution, however, must be taken when interpreting the sites of presumed penetration. It is possible to achieve similar results in the absence of bacteria (Fig. 3.6b). In this case, the absence of bacteria is implied by the intact extracellular matrix, which, as previously demonstrated, is eroded by the bacteria. The effects on the hyphal cell are similar to those described previously, the underlying plasma membrane having become diffuse close to the site of the breach, however, the plasma membrane appears to be distorted, protracted and folded back on itself, possibly as a result of a hypersensitive or wound response or repair mechanism. It is important to note the possibility that aberrations may occur due to faults in the resin. In Fig. 3.5a, two of the three wall breaks have an associated dark area seemingly unrelated to the fungal wall. These areas were seen to develop when exposed to the electron beam in the microscope. Whether these breaks were caused by the resin expanding or reacting with the electron beam causing a tear in the fungal wall is unknown. The third breach apparently remains clear.

Often, the intercellular space, previously consisting of the extracellular matrix, becomes full of microfibrils which are thought to be of bacterial origin. Their exact nature, however, is unknown (Fig. 3.6c). The vesicles which are also found in the intercellular space are definitely bacterial in origin. These migrate to the fungal wall and deliver their contents against the wall (Fig. 3.6d). Their presence at the wall coincides with a disruption in the integrity of the outer polysaccharide layers, indicated by a release of microfibrils (Fig. 3.7a). In some cases, the vesicles appear to interact intimately with fungal wall components (Fig. 3.7b). The identity of the microfibrils interacting at the fungal wall - extracellular interface is, however, uncertain. They may be bacterial appendages (flagella, fimbriae or pili) intertwined with fungal glucan microfibrils, or they may in fact be chitin microfibrils (Fig. 3.7c). The bacteria themselves also interact closely with the fungal wall, inducing swellings of the wall to develop into the intercellular space (Fig. 3.7d).

3.3.3 Colloidal Gold - Wheat Germ Agglutinin Labelling of Fungal Wall Chitin

The control treatments employed to test the efficacy and specificity of the colloidal gold-wheat germ agglutinin labelling system provide good support for lectin-chitin and Ovomucoid-lectin specificity and the reliability of each facet of the system.

In the absence of wheat germ agglutinin, fungal walls are devoid of colloidal gold (Fig. 3.8a). This demonstrates the inability of the Ovomucoid-gold conjugate to bind directly to N-acetylglucosamine and is indicative of the role played by wheat germ agglutinin in recognizing and binding to N-acetylglucosamine residues.

The specificity exhibited by Ovomucoid for wheat germ agglutinin is expressed in the second control (Fig. 3.8b) in which wheat germ agglutinin, bound to fungal wall chitin, has all its binding sites 'satisfied' by incubation with Ovomucoid. The inability of the Ovomucoid-gold conjugate to subsequently bind to the 'satisfied' wheat germ agglutinin indicates the preference these two proteins have for each other.

The colloidal gold-wheat germ agglutinin labelling system has the ability to distinguish between two major skeletal polysaccharides, chitin and cellulose (Fig. 3.8c). The gold binds preferentially to the fungal wall which lies adjacent to the cellulosic plant wall. Despite some background labelling, the density of gold is notably much greater over the chitinous *Rhizopogon* hyphal wall than the plant wall.

The Oomycetes provide an unusual situation in so far as some members of this group (such as *Saprolegnia ferax*) possess chitin as the major skeletal polysaccharide, while others (*Phytophthora nicotianae*) have a cellulosic skeletal polysaccharide. This can be demonstrated with the aid of the colloidal gold-wheat germ agglutinin labelling system (Figs. 3.9a, 3.9b)

which shows gold particles overlying the *Saprolegnia* wall as opposed to the *Phytophthora* wall.
Once all the intricacies of the system have been resolved, and the labelling protocol applied to unadulterated *Agaricus* hyphal walls, gold particles may be seen evenly distributed along the walls (Fig 3.8d).

Evidence to support the specificity of this labelling system has been presented, however, the reliability and reproducibility of labelling is equally important. The analysis of labelling density revealed no significant variation ($P<0.05$) in gold density between the two grids stained separately in different wells (Appendix C). This is despite the inclusion of a washing step which varies in intensity depending upon the user. This washing step is critical in the labelling system. It removes unbound wheat germ agglutinin remaining on the section, which would be targeted by the ovomucoid-gold conjugate. That this variation is not reflected in the density of gold on fungal wall sections suggests unbound wheat germ agglutinin is removed early in the wash with little difficulty. The analysis of gold particle density on walls both in the presence and absence of bacteria (Appendix D) has shown that bacteria have no significant effect on the level of binding ($P<0.05$) and therefore implies that the amount of chitin in the walls remains unaffected.

Gold labelling of fungal wall chitin was used to characterize some of the features of the bacterial/fungal interaction visualized with uranyl acetate-lead citrate staining. The exposed faces of broken walls were examined and appeared more typical of a mechanical break rather than an enzymatic degradation. Gold particles are seen to bind to the exposed face suggesting that the integrity of the skeletal chitin/ß-glucan matrix has been maintained (Fig. 3.10a). In this same example, a darkening of the resin is seen, implying this break may not be induced by bacteria. A closer investigation of the ends of a break show them to be abrupt and the gold label continues to the exposed faces (Fig. 3.10b). Bacterial cells in close proximity to fungal walls affect the outer glucan layers (Fig. 3.10c) but as the gold labelling shows, the underlying chitin layer is not disturbed, gold labelling being absent from the swelling. Indeed, it appears the chitin layer remains totally unaffected by the presence of bacteria (Fig. 3.10d). In Fig 3.11a, the fungal wall appears intact despite the close proximity of the

bacterial cell. Gold particles on the edge of the exposed surface may be the result of the outer glucan layers having been removed. This is also the case in Fig. 3.11b. Chitin is again prevalent near the exposed bacterial/fungal interface, though the plasma membrane appears to be intact and unaffected, unlike that in Fig. 3.11a. The microfibrils, associated with the presence of bacteria, seen in previous uranyl acetate-lead citrate stained sections (Fig. 3.7c), did not respond positively to colloidal gold-wheat germ agglutinin labelling (Fig. 3.11d). This indicates that they are not chitin microfibrils released from the walls during possible wall degradation. A similar result was obtained for the blebs or vesicles attached to the outer bacterial envelope (Fig. 3.11c). In these three cases, the fungal wall and its chitin complement remains intact despite the close proximity of bacterial cells. Even when bacterial cells appear to be adherent to fungal hyphae (the crenulate outer bacterial membrane becoming indistinguishable from the hyphal wall layers), the chitin layers remain unaffected (Fig. 3.11d). The particulate gold labelling appears quite heavy toward the outer area of the fungal wall, again suggestive of a removal of the outer glucan layers.

3.3.4 Detection of Glucan Degradation

Thiery's test for polysaccharides clearly indicated a three-layered fungal wall structure (Fig. 3.12a). In the presence of the 'drippy gill' bacterium, the outer layer appears to be affected (Fig 3.12b). There is a loss of integrity of this wall layer, though underlying components remain unaltered. Even when breaks in the wall appear, the broken ends do not show evidence of enzymic degradation (Fig. 3.12c), rather they are characteristic of being broken mechanically. However, the presence of a bacterial cell is indicated (Fig 3.12c) by the remnant of a microfibril, at the mouth of the breach. Also, the upper portion of fungal wall shows some evidence of disruption, by the 'looseness' of the outer layer packing and the absence of the darker internal layer which is visible in the lower wall section. That the bacterium has the ability to disrupt the outer glucan layer can be infered (Fig. 3.12d) by the way this layer is pulled back and the

interconnecting glucan microfibrils remain behind. There is also evidence of a profusion of bacterial blebs or vesicles throughout the intercellular space, some of which appear at the fungal wall-extracellular interface. These protuberances indicate that they possess an outer membrane as demonstrated by the positive reaction to Thiery's stain (Fig. 3.13a). The membranes are continuous with that of the parent bacterial outer membranes lending support to their origins (Fig. 3.13b). The vesicles are released from the bacterium (Fig. 3.13c) and migrate to the target hyphal wall against which they deposit their contents (Fig. 3.13d). The end result of the vesicular migration is a disruption of the fungal wall outer glucan layer, resulting in the protrusion of wall microfibrils into the intercellular space as seen previously (Figs. 3.7a, 3.7c). The composition of these microfibrils is predominantly polysaccharide, as indicated by the positive reaction to Thiery's stain (Fig. 3.14).

'Drippy gill' bacteria seem to possess a number of 'appendages'. *P. agarici* and PV29 are both multiflagellate (Fig. 3.15a), they have a number of short appendages emanating from the outer membrane (Figs. 3.15b, 3.15c) and a plethora of shorter peg-like extensions (Fig. 3.15d). The shorter appendages (Figs. 3.15b, 3.15c) appear to be sectioned flagella. Gold particles do not bind to them indicating they are not chitinous and are therefore not overlying chitin microfibrils possibly derived from degrading fungal walls. Their common point of attachment to the bacterium, their number and indistinct Thiery reaction, suggests these appendages are flagella. The appearance of a further portion of the same appendage at a distance from the bacterium appears to confirm this, as flagella would tend to pass in and out of the plane of section (Fig. 3.15c). The ruthenium red preparations (Figs. 3.16a, 3.16b) demonstrate aggregations of polysaccharide at the surface of the outer bacterial membrane which may correlate with the peg-like structures seen previously (Fig. 3.15d).

The use of Clinistix to detect the evolution of glucose from three glucan substrates as a result of the action of a glucanase enzyme produced by the 'drippy gill' organism, indicated that both *P. agarici* and PV29 were negative in this respect (Table 3.3). PMS164 produced glucose from laminarin at a level in excess of 14mmol and functions as a positive control

for ß-glucanase production. The results generated were identical for both the constitutive and the inducible enzyme assays.

The mean reducing sugar concentrations yielded from the glucose standard concentration curve via the HBH method (Table 3.4), parallel the findings of the Clinistix assay. In the case of laminarin, ANOVA reveals a significant variance among the results (Appendix E). The concentration of reducing sugar produced by PMS164 from laminarin is highly significantly different ($P<0.001$) from the remaining three treatments, among which there is no significant variation. Therefore, the two 'drippy gill' organisms are unable to hydrolyse laminarin. The concentration of reducing sugar in these treatments is the same as found in a 0.3% solution of laminarin in nutrient broth, possibly due to some spontaneous dissociation of the glucan.

In the case of nigeran, an insoluble glucan, the HBH method and subsequent ANOVA reveals a significant variation between the test organisms in their ability to hydrolyse the substrate (Appendix F). *P. agarici* generated less reducing sugar than the other three treatments, including the control. This spurious result does not seem relevant in this case, as it is unlikely that a positive glucanase assay would result in a reduction of detectable sugar.

The HBH detection of reducing sugar evolved from pullulan and the ANOVA of the results reveals that the two 'drippy gill' organisms and PMS164 are unable to hydrolyse this glucan, indicated by the fact that there is no significant variation between the treatments and the control (Appendix G).

Table 3.3 Glucose[a] Production from Various Glucans

	GLUCAN SUBSTRATE		
Isolate	Laminarin	Nigeran	Pullulan
P. agarici	-	-	-
PV29	-	-	-
PMS 164	+	-	-
NB + Substrate	-	-	-

[a] Presence of glucose determined by Clinistix

Table 3.4 Reducing Sugar[a] Determination - HBH Method

	GLUCAN SUBSTRATE		
Isolate	Laminarin	Nigeran	Pullulan
P. agarici	7.783	1.24	0.966
PV29	6.725	1.299	0.944
PMS 164	16.3	1.292	1.03
NB + Substrate	7.029	1.312	1.054

[a] Mean sugar concentration expressed as mmol glucose

3.3.5 Isolation and Digestion of *Agaricus* Wall Fractions

The 'drippy gill' organisms and the positive ß-glucanase control bacterium were unable to liberate a detectable level of glucose (using Clinistix) from any of the three isolated wall fractions. The assay involving Reese and Mandel's buffered glucan solution and Clinistix proved positive for the PMS164 control only, demonstrating that the 'drippy gill' bacteria were unable to produce ß-glucanase or α-glucanase to hydrolyse the fractions.

3.3.6 The Enzymatic Degradation of *Agaricus* mycelium

Control - Gold Label: The labelling intensity of the chitin in *Agaricus* vegetative mycelium from plate culture is very much reduced from that seen in sporocarp tissue, which reflects the decreased chitin content in secondary mycelium walls (Fig. 3.17a). Also very noticeable are the extensive wall breaks which are also prevalent throughout the treated tissues.

Control - TCH: In this particular hyphal section (Fig. 3.17b), the wall remains distinct from the plasma membrane, also shown to stain positively. A variation of wall integrity is marked.

ß-Glucanase - Gold Label: ß-glucanase treatment results in gold label being confined to a narrow band of darkly opaque hyphal wall (Figs. 3.18a, 3.18b). Despite the low density of the gold particles, the labelling indicates that the chitin layer remains largely unaffected by ß-glucanase treatment and is comparable to the controls. Breaks in the wall are prolific and again mirror those seen in the control sections. The outer wall layers appear to be diffuse, implying a degree of hydrolysis of the outer wall glucans. The wall microfibrils, liberated as a result of the enzymatic attack on outer glucans, react negatively to gold labelling, indicating they are not chitin microfibrils (Fig. 3.18c).

ß-Glucanase - TCH: Tch staining of ß-glucanase treated *Agaricus* hyphal walls reveals damage to the outer glucan layers and many wall discontinuities (Fig. 3.19a). Microfibrils released from the fungal wall into the extracellular space (Fig. 3.19b), react positively to Thiery's stain confirming they are composed of polysaccharides (Fig. 3.19c). A closer examination of protruding microfibrils revealed they are continuous with the hyphal wall (Fig. 3.19d) and are therefore derived from it. Thiery's staining also reveals damage to the underlying fungal cytoplasmic membrane, exposed to ß-glucanase perhaps through the wall breaches. The membrane appears to have spread laterally and become detached from the fungal wall. The damage caused to fungal walls by the 'drippy gill' bacteria closely resembles the damage caused by ß-glucanase treatment.

Chitinase - Gold Label: The distribution of gold label over chitinase treated *Agaricus* vegetative mycelium indicates that chitin is still present in the walls (Fig. 3.20a). The outer glucan layers appear to have been dissipated, but the inner chitinous layer remains intact. Seemingly less densely packed than in untreated walls, the density of gold label over the inner wall, though low (Fig. 3.20b), indicates that chitin is still present and has not been completely hydrolysed, if at all.

Chitinase - TCH: Hyphal walls remain largely intact, though breaks, also visualized in control sections, are again in evidence. The outer glucan layers appear to be affected (Fig. 3.21a) as shown by a dark trace approximately parallel to and 20-30nm from the main body of the remaining wall. The breach (Fig. 3.21b) has abrupt ends, suggestive of a mechanical breakage. Wall-derived microfibrils, seen in glucanase treatments, are absent, indicating the damage to the wall is less severe than previously encountered (Fig. 3.19b). Aggregates of TCH positive material commonly form both inside and outside treated walls (Fig. 3.21c).

ß-Glucanase - Chitinase - Gold Label: Vegetative mycelial walls of *Agaricus* treated with ß-glucanase followed by chitinase, show a complete degradation of the hyphal wall (Fig. 3.22a). The complete absence of gold label from the remnant of wall, evident in section as a trace only, indicates the complete hydrolysis of the wall chitin. Even when some structure

remains of the digested wall, it is not able to be labelled with gold, again indicating the absence of chitin.

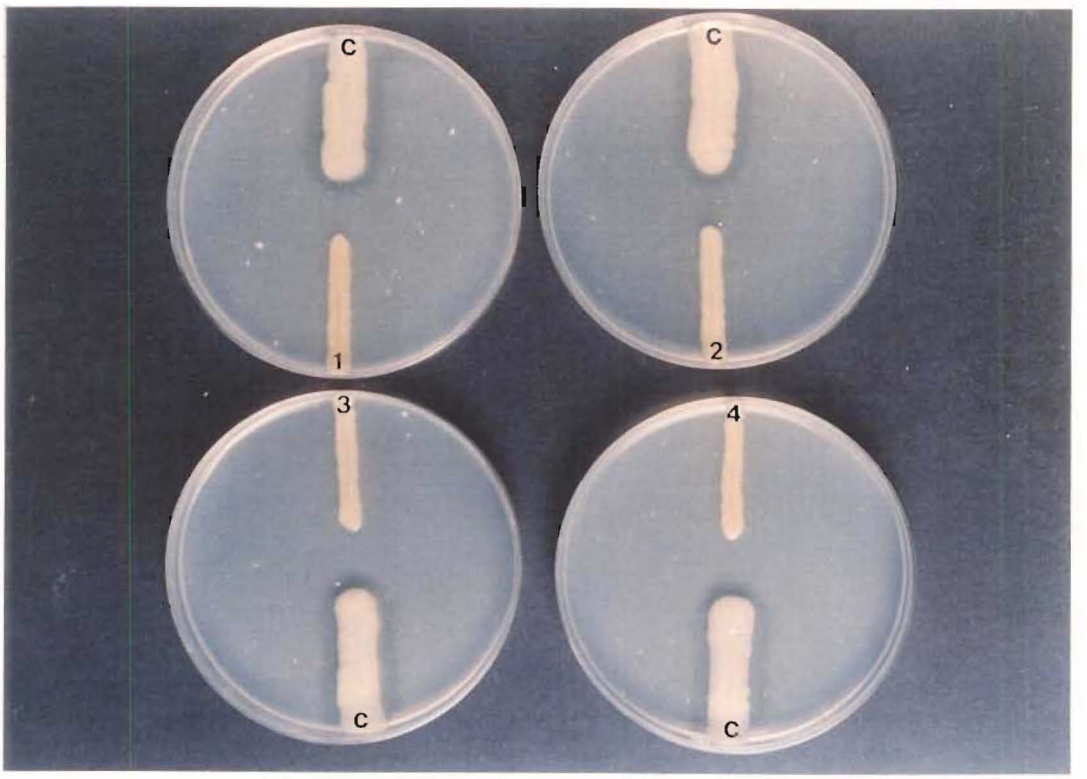
ß-Glucanase - Chitinase - TCH: The sections of wall which react positively to Thiery's stain are very much reduced. The majority of the hyphal walls have been hydrolysed and are evident only as a trace on the section (Fig. 3.23a) and discontinuities in the wall are prevalent. A relatively intact section of wall demonstrates the pattern expected in an enzymatic digestion of wall material (Fig. 3.23b), where the exposed ends of a breach are not ragged or abrupt, but show a decreasing density of wall material approaching the break. Interestingly, it appears that the inner wall layer, the site of hyphal wall chitin, is discontinuous at different places to the outer glucan layers, a situation very different to the breaches seen previously and attributed to an external mechanical force.

3.3.7 Adhesion Assay

The adhesion assay yielded similar results for both *P. agarici* and PV29 in their ability to adhere to *Agaricus* mycelium. The recovered PV29 bacteria revealed an adhesion rate of 74.4% and for *P. agarici*, 81.5% adhered firmly to the mushroom mycelium.

FIGURE 3.2 'Drippy gill' isolates on standard chitin agar. Note the haloes of clearing around the positive control (C), *S. liquefaciens*. 1=PMS601; 2=PMS603; 3=PV29; 4=PMS752.

FIGURE 3.3 'Drippy gill' isolates on chitin agar amended with mushroom extract. Note the haloes of clearing around the positive control (C), *S. liquefaciens*. 1=PMS601; 2=PMS603; 3=PV29; 4=PMS752.


C
C
1
2
3
4
C
C

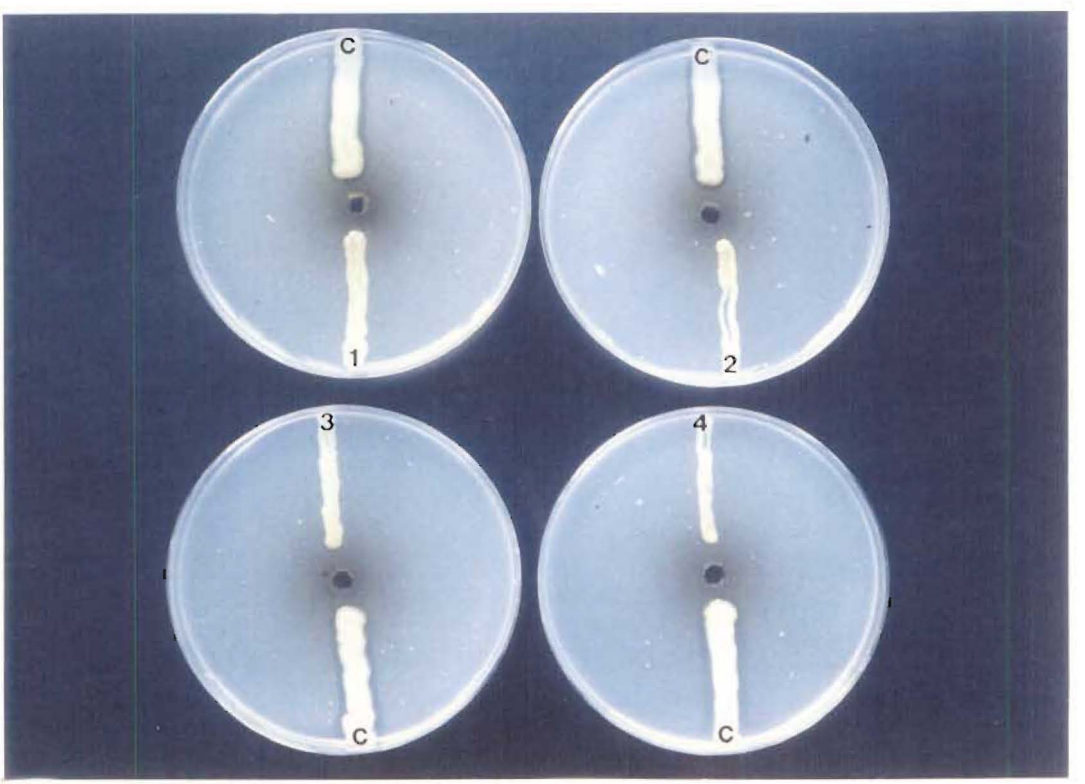

C
C
1
2
3
4
C
C

FIGURE 3.4

Fig. 3.4a 'Drippy gill' bacteria in *Agaricus* hymenial tissue. The majority of bacteria are extracellular, though some are intrahyphal (arrow). Bar=2μm.

Fig. 3.4b 'Drippy gill' bacteria in *Agaricus* hymenium. The presence of bacteria is associated with a degradation of the extracellular matrix, the remnants of which are indicated (EM). Note the disruption of the hyphal walls (arrow). Bar=2μm.

Fig. 3.4c *Agaricus* hyphal extracellular matrix. Degradation of the extracellular matrix (EM) seemingly occurs in the absence of bacteria. However, portions of bacterial flagella can be seen within areas of clearing (arrow). Bar=2μm.

Fig. 3.4d 'Drippy gill' affected tissue in an *Agaricus* sporocarp. The hyphal wall (FW) is apparently being degraded. There is evidence of an intrahyphal bacterial cell (arrow). Note the lack of cellular organisation within the fungal cell. Bar=500nm.

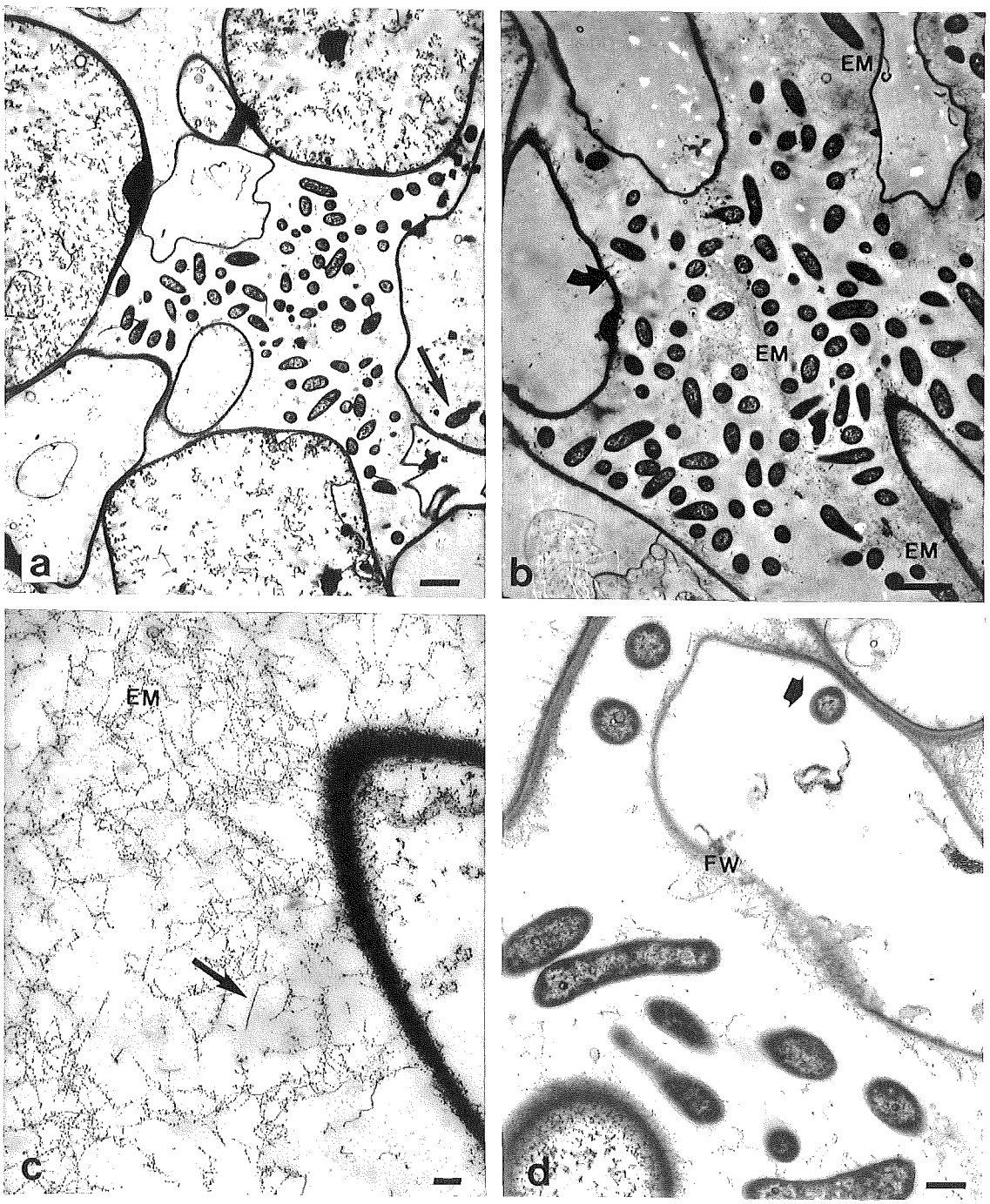

a
b
EM
EM
EM
c
EM
d
FW

FIGURE 3.5

Fig. 3.5a Multiple breaches of a fungal cell wall in a 'drippy gill' affected *Agaricus* sporocarp. Note the intact extracellular matrix (EM) between the bacterium and wall break. The fungal plasma membrane (PM) appears to be disrupted. The discolouration of the resin associated with the other two breaks (arrows) indicates an aberration caused by resin disturbance. Bar = 50nm.

Fig. 3.5b *Agaricus* hyphal wall breaks with associated 'drippy gill' bacteria. Again notice the loss of integrity of the fungal plasma membrane (PM). Bar = 500nm.

Fig. 3.5c 'Drippy gill' bacteria and associated hyphal wall breach. The fungal plasma membrane (PM) again appears to have lost its integrity compared with the plasma membrane of the adjacent fungal cell (arrow). Bar = 200nm.

Fig. 3.5d 'Drippy gill' bacterium entering fungal cell, already occupied by bacteria. Note the lack of cellular organisation within the occupied fungal cell. Bar = 1μm.

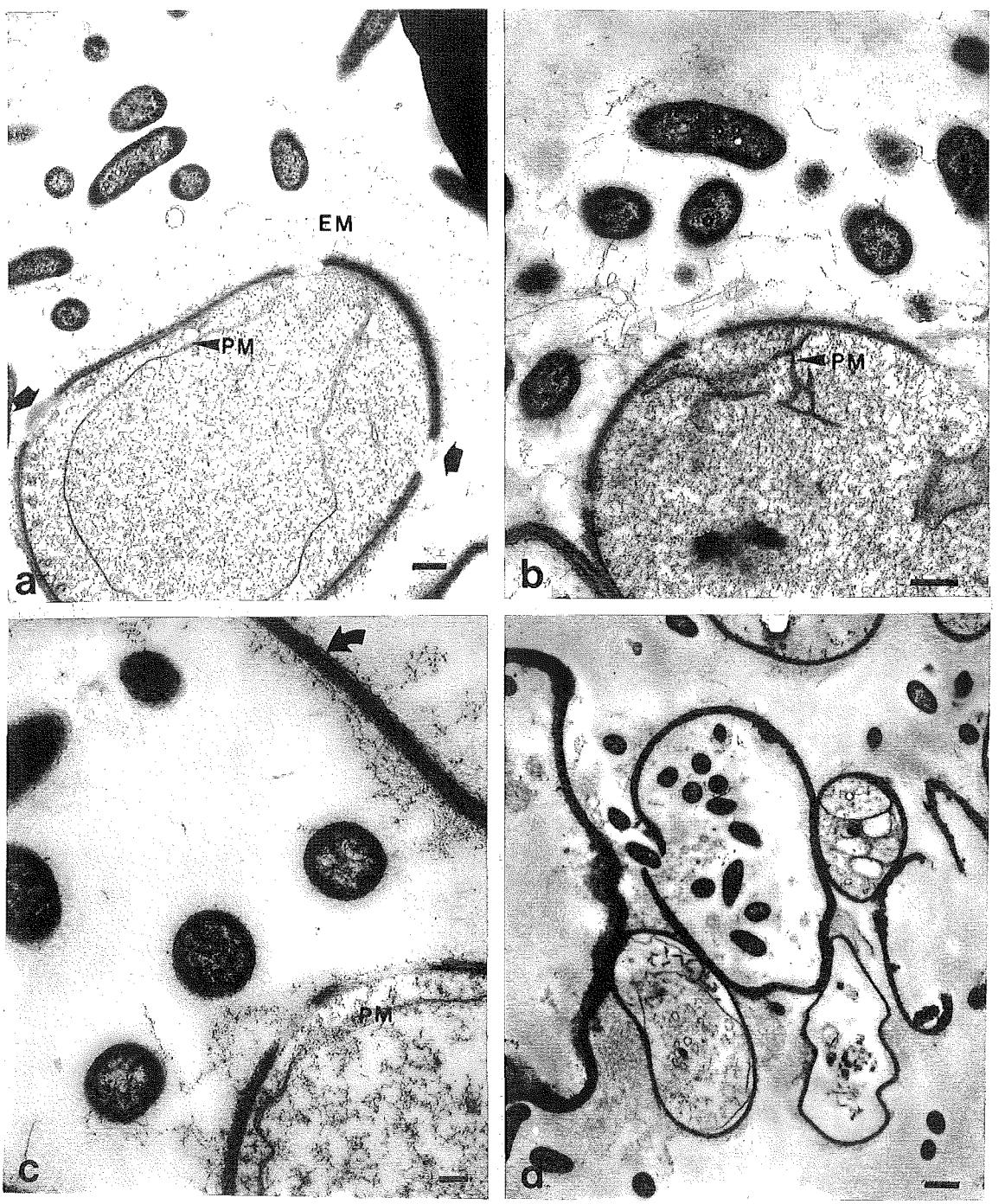

EM
PM
a
PM
b
PM
c
d

FIGURE 3.6

Fig. 3.6a Site of penetration of an *Agaricus* hyphal cell (FC) by a 'drippy gill' bacterium. The exposed ends of the broken fungal wall appear ragged as though mechanically parted. Bar=200nm.

Fig. 3.6b Hyphal wall breaks in a 'drippy gill' afflicted sporocarp. These may be observed without the presence of bacteria. The intact extracellular matrix (EM) indicates the absence of bacteria. Bar=200nm.

Fig. 3.6c Bacteria and associated large microfibrils, presumed to be flagella, in the hyphal intercellular space of an *Agaricus* sporocarp. The proximity of bacteria is often indicated by remnants of flagella in a particular section. Note also the profuse vesicles (arrow) on the surface of the bacterial outer envelope. Bar=200nm.

Fig. 3.6d 'Drippy gill' bacteria within the *Agaricus* hyphal intercellular space. The vesicles migrate from the bacterium to the hyphal wall (arrow). Note the prevalent microfibrils and lack of extracellular matrix. Bar=200nm.

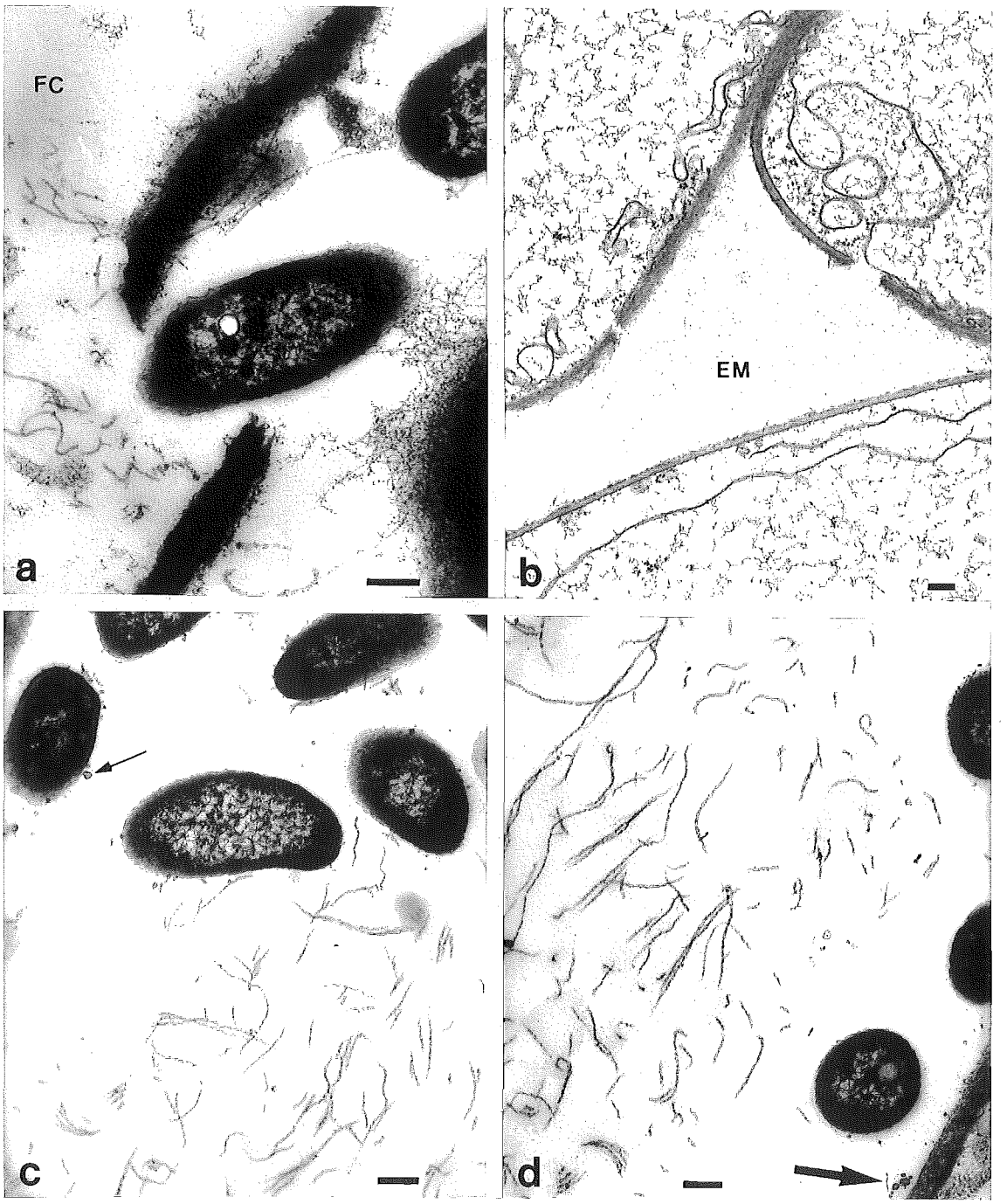

FC
a
EM
b
c
d

FIGURE 3.7

Fig. 3.7a 'Drippy gill' bacterium (DG) adjacent to an *Agaricus* hyphal wall (FW). Bacterial vesicles (BV) are associated with release of fungal wall-derived microfibrils (FM). Bar=50nm.

Fig. 3.7b 'Drippy gill' bacterium (DG) in close association with an *Agaricus* hyphal wall (FW). There appears to be an intimate association between some vesicles (BV) and fungal wall components. Bar=50nm.

Fig. 3.7c An *Agaricus* hyphal wall showing a disruption to its outer layers. The fungal wall (FW) releases a profusion of microfibrils as a result of bacterial influence. Bar=100nm.

Fig. 3.7d *Agaricus* hyphal wall (FW) and a 'drippy gill' bacterium (DG). Not all interactions between bacteria and fungal cells appear to be dependent upon the migration of vesicles to the fungal wall. Bar=50nm.

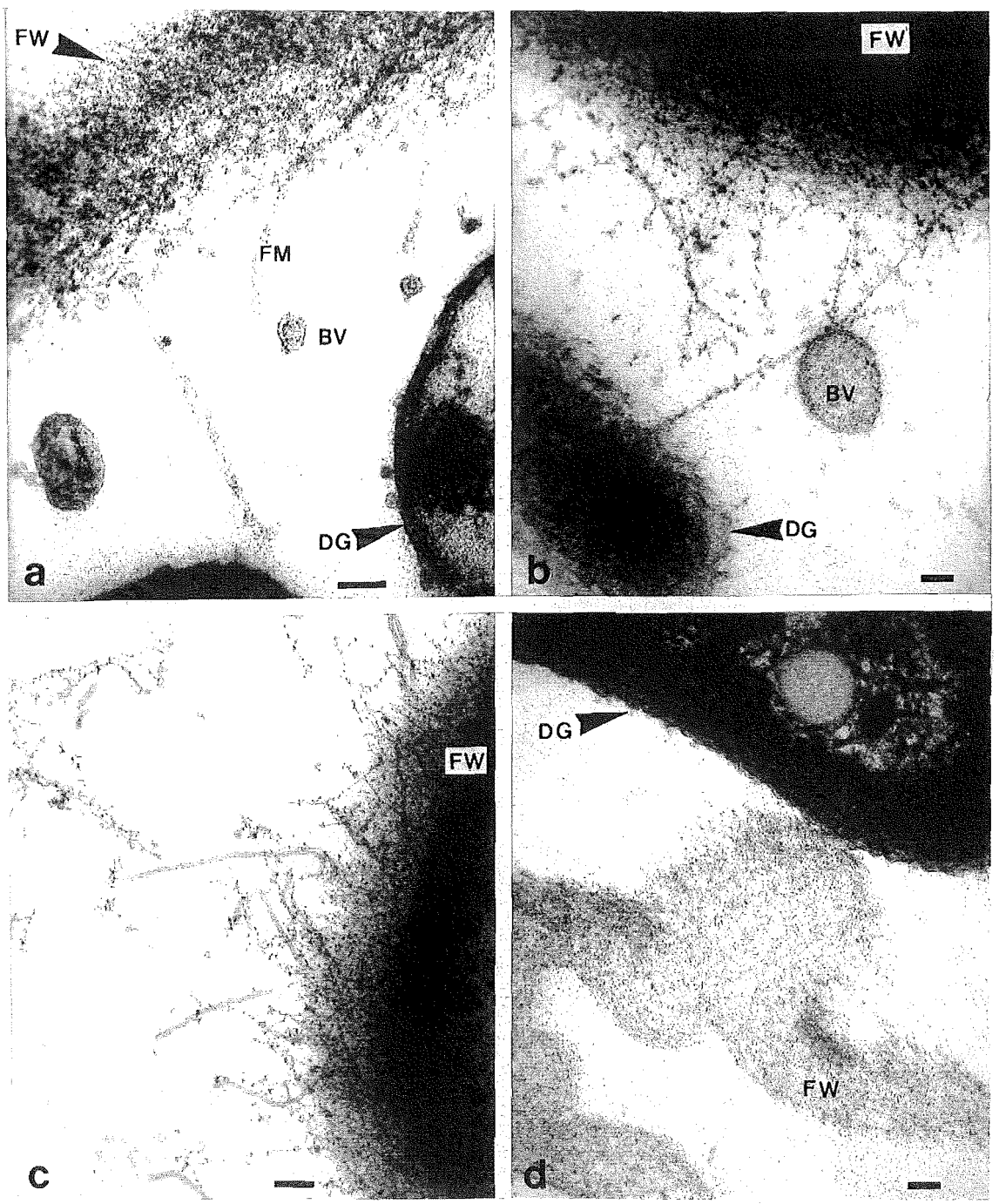

FW
FM
BV
DG
a
FW
BV
DG
b
FW
c
DG
FW
d

FIGURE 3.8

Gold Labelling Controls

FC = fungal cytoplasm, IS = intercellular space

Fig. 3.8a Colloidal gold labelling control. The *Agaricus* hyphal wall (FW), being devoid of gold particles, demonstrates the specificity of the Ovomucoid-gold complex for wheat germ agglutinin and its inability to bind directly to N-acetylglucosamine. Bar = 200nm.

Fig. 3.8b Colloidal gold labelling control. The *Agaricus* hyphal wall (FW), being devoid of gold particles, demonstrates that gold requires Ovomucoid to mediate binding to wheat germ agglutinin and subsequently N-acetylglucosamine. Also indicated is the specificity Ovomucoid has for wheat germ agglutinin. By filling all available binding sites, Ovomucoid prevents the Ovomucoid-gold complex binding to wheat germ agglutinin. Bar = 50nm.

Fig. 3.8c *Rhizopogon* mycorrhizal association with *Pinus*. The *Rhizopogon* fungal wall (FW) is labelled with gold particles. The *Pinus* cell wall (PW) is not labelled with gold indicating the preferential binding of gold to chitin, over another glucose polymer, cellulose. Some background staining is visible (arrow) outside the plant wall. Bar = 200nm.

Fig. 3.8d *Agaricus* hyphal wall treated with wheat germ agglutinin and Ovomucoid-gold complex. This treatment demonstrates the labelling of chitin. Note the low level of background labelling. Bar = 200nm.

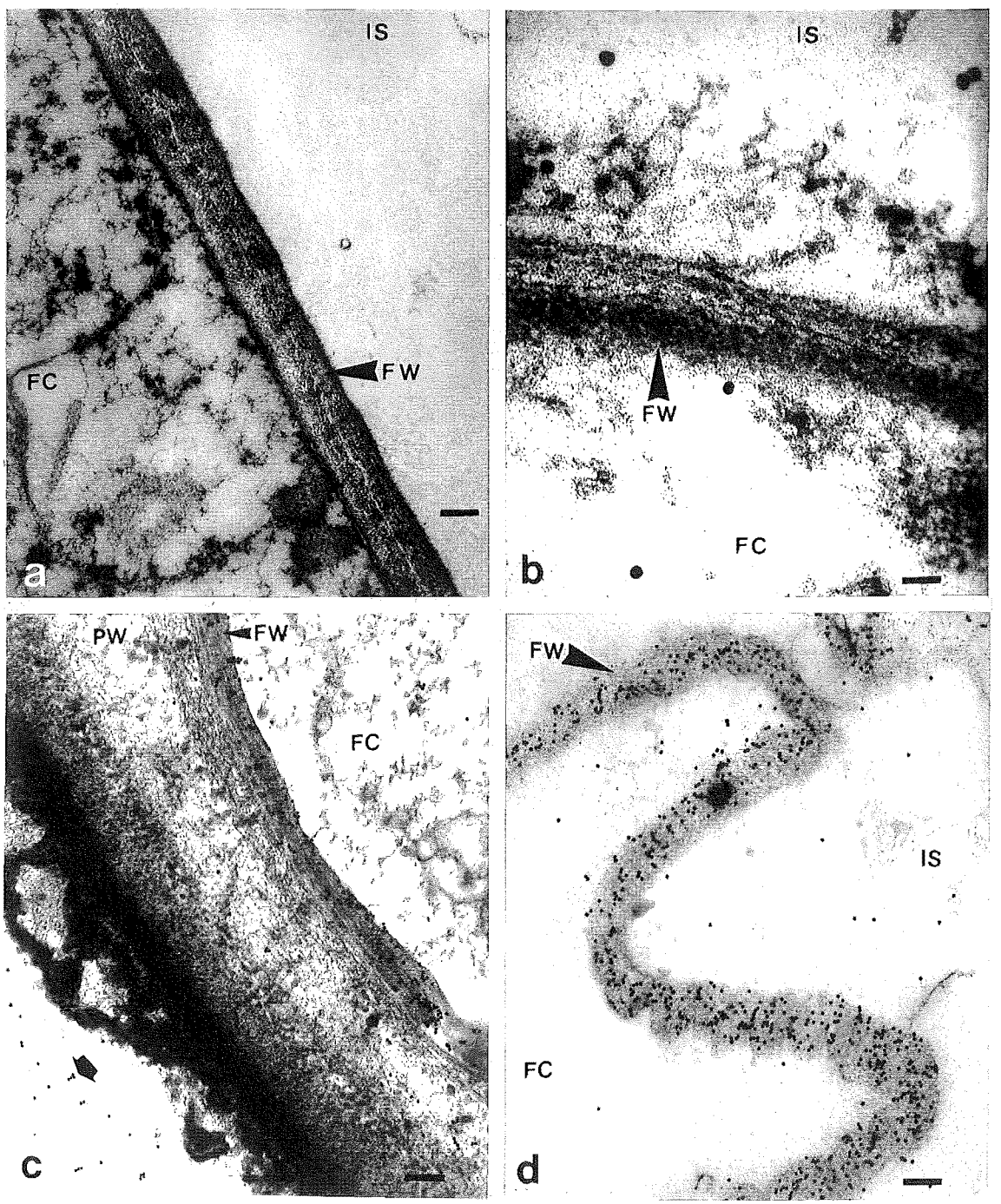

IS
FC
FW
a
IS
FW
FC
b
PW
FW
FC
c
FW
IS
FC
d

FIGURE 3.9

Fig. 3.9a Hyphal wall (FW) of the Oomycete *Phytophthora nicotianae*. The absence of chitin in the *Phytophthora* wall is indicated by the lack of gold particles binding to the walls following colloidal gold labelling. Bar = 100nm.

Fig. 3.9b *Saprolegnia ferax* hyphal wall (FW). This is an Oomycete which does possess chitin as a structural polymer, demonstrated by the profusion of gold particles binding to the fungal walls following colloidal gold labelling. FC - Fungal cytoplasm. Bar = 100nm.

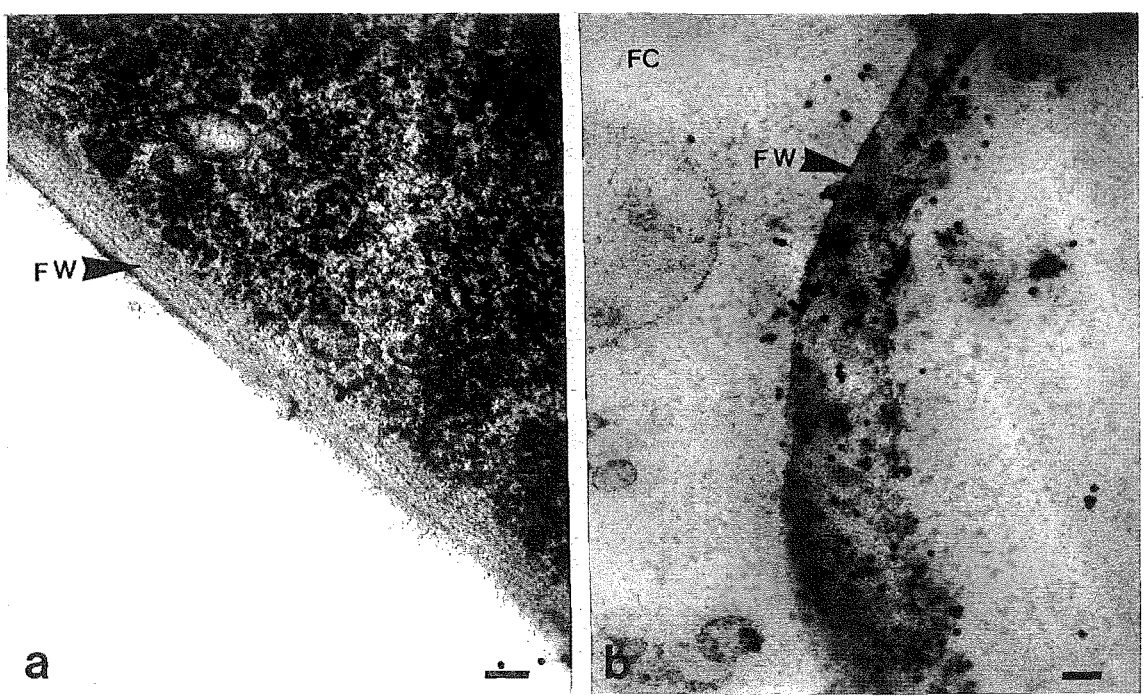

FW
a
FC
FW
b

FIGURE 3.10

Fig. 3.10a *Agaricus* hyphal wall from a 'drippy gill' afflicted sporocarp showing a wall breach (possibly an artefact caused by the distortion of the embedding resin, seen as a dark area (arrow) at the entrance of the break). Gold labelling demonstrates that chitin is not degraded. Note the bacterial flagella. Bar=200nm.

Fig. 3.10b Wall break in an *Agaricus* hyphal cell. Wall breaks remote from the influence of resin distortion show their exposed ends to be ragged in appearance. Again, gold labelling demonstrates that chitin has not been degraded at the site of the breach. Bar=50nm.

Fig. 3.10c 'Drippy gill' bacterium in close proximity to an *Agaricus* hyphal wall. Induction of glucan swelling, indicated as area lacking gold particles (arrow), by bacterial cell. Note the appendages on the bacterial surface. Bar=100nm.

Fig. 3.10d 'Drippy gill' bacteria in *Agaricus* hymenial tissue. The bacteria occur extracellularly. Note the high degree of background labelling due to the age of the Ovomucoid-gold conjugate. No breaches of the hyphal walls are visible, which are heavily labelled despite the age of the reagent. Bar=1μm.

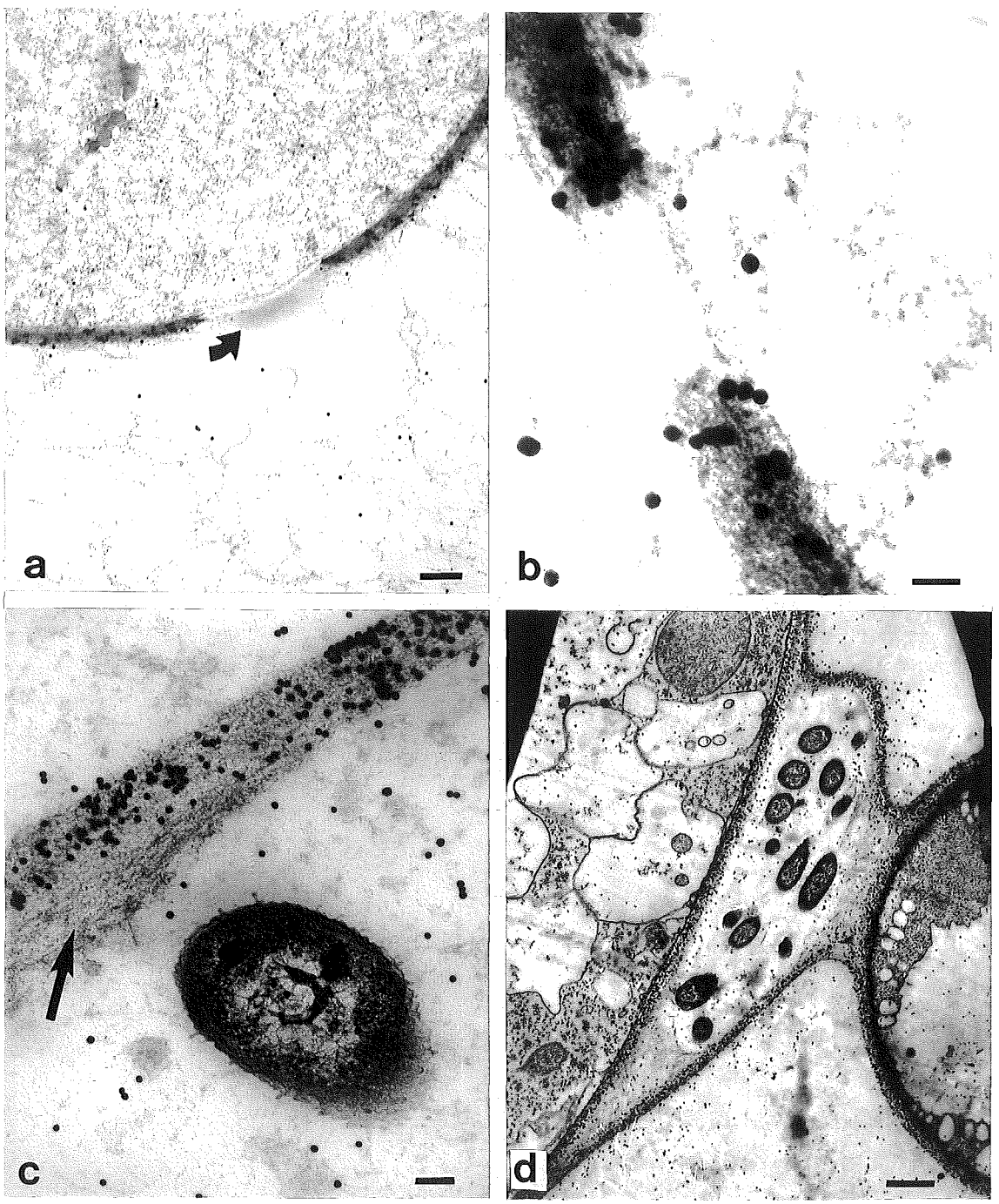

a
b
c
d

FIGURE 3.11

FC = fungal cytoplasm, IS = intercellular space

Fig. 3.11a *P. agarici* adjacent to an *Agaricus* hyphal wall (FW). Gold labelling, evident in close proximity to the bacterial cell (DG), indicates that chitin is unaffected by the bacterium. Note the lack of fungal plasma membrane and structure within the fungal cytoplasm. Bar = 100nm.

Fig. 3.11b *P. agarici* in close proximity to an *Agaricus* hyphal wall. There appears to be no disturbance in the chitinous layer of the fungal wall (FW) despite the close proximity of a bacterial cell (DG). In this case, the fungal plasma membrane is intact (arrow). Bar = 100nm.

Fig. 3.11c PV29 adjacent to an *Agaricus* wall (FW). Gold labelling again indicates the chitin layer to be unaffected by the presence of the bacterial cell (DG). Bar = 100nm.

Fig. 3.11d 'Drippy gill' bacterial cell (DG) between two *Agaricus* hyphal walls (FW). Both walls appear to have unaffected chitin layers despite the apparent adherence of the bacterial cell to the walls. Note the intact hyphal organization within the fungal hypha (FH). Bar = 100nm.

Fig. 3.11e Microfibrils (MF) derived from the *Agaricus* hyphal wall (FW). These fail to provide binding sites for colloidal gold indicating their non chitinous nature. Bar = 100nm.

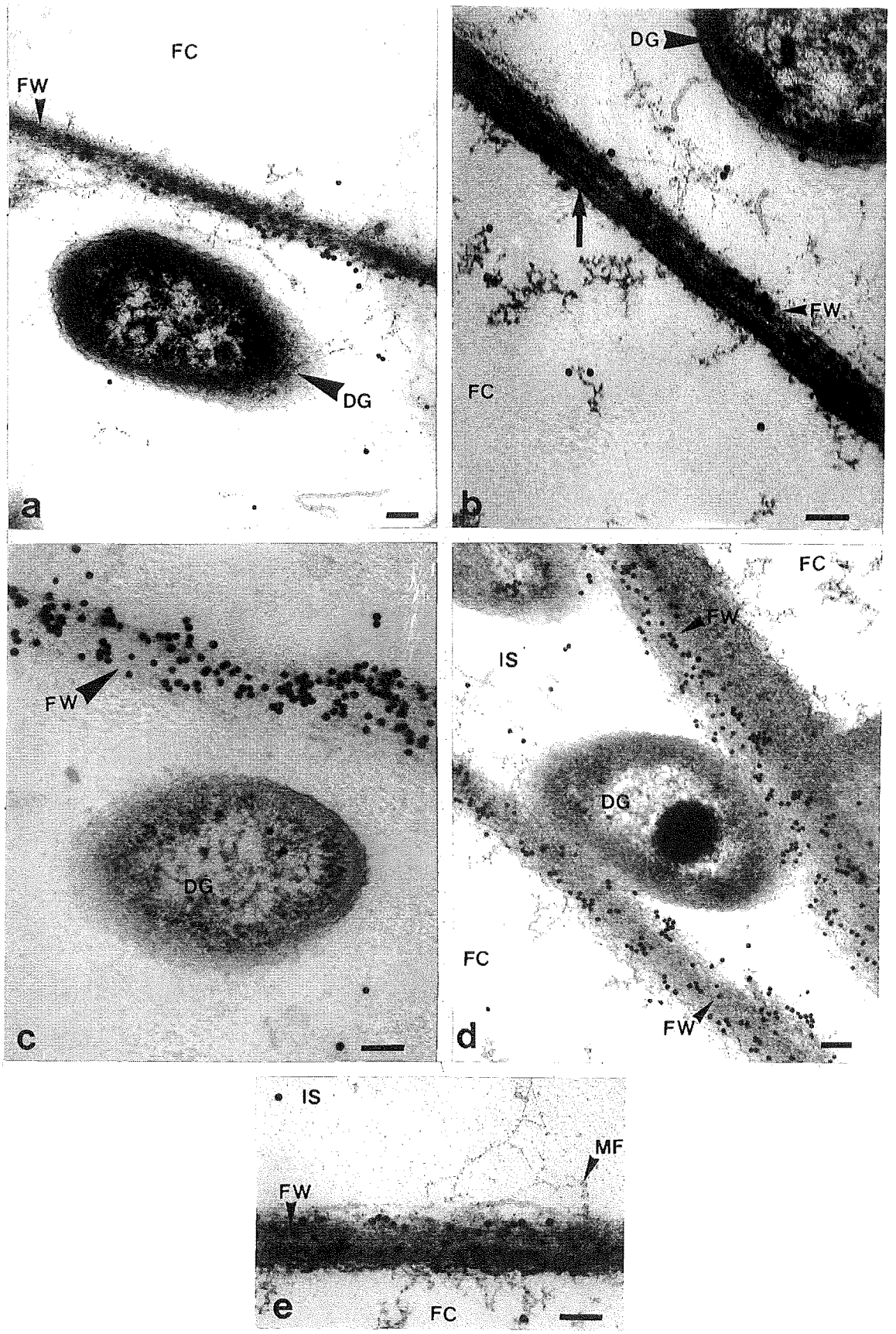

FC
FW
DG
a
DG
FW
FC
b
FW
DG
c
FC
FW
IS
DG
FC
FW
d
IS
MF
FW
FC
e

FIGURE 3.12

FC = fungal cytoplasm, IS = intercellular space

Fig. 3.12a *Agaricus* hyphal wall (FW) stained with TCH. This preparation shows the multilayered polysaccharidic wall. Bar=200nm.

Fig. 3.12b *Agaricus* hyphal wall (FW) and adjacent 'drippy gill' bacterial cell (DG). Note the bacterial extracellular polysaccharide obscuring the crenulate outer bacterial envelope. There also appears to be a disruption to the outer polysaccharide layer of the fungal wall. Bar=50nm.

Fig. 3.12c An *Agaricus* hyphal wall break. This break has occurred in proximity to a bacterial cell (indicated by the flagella remnant - FR). Note the ragged exposed ends and the continuation of the wall layers. The top section appears to have undergone some alteration or disturbance and has had the fungal plasma membrane removed from it. Bar=100nm.

Fig. 3.12d 'Drippy gill' bacteria and *Agaricus* wall (FW). Note the vesicles of bacterial origin in the intercellular space. The outer layer of the fungal wall has seemingly been peeled away (arrow) by the adjacent bacterial cell. Bar=200nm.

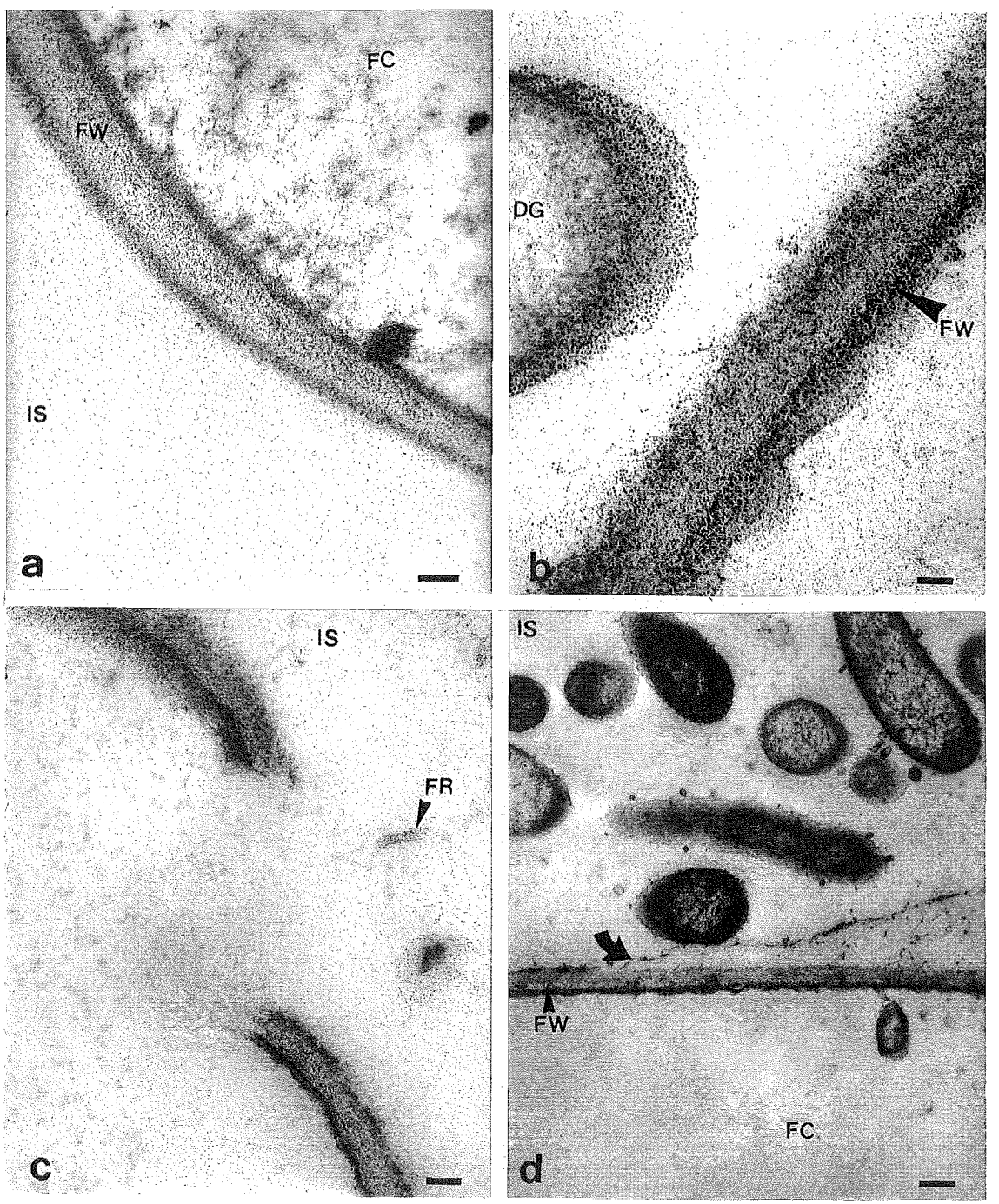

FC
FW
IS
a
DG
FW
b
IS
FR
c
IS
FW
FC
d

FIGURE 3.13

FC = fungal cytoplasm, IS = intercellular space

Fig. 3.13a A 'drippy gill' bacterium (DG) with associated vesicle. The vesicle (V) possesses a membrane which gives a positive reaction for polysaccharide by Thiery's staining. Note the close proximity of the *Agaricus* hyphal wall (FW). Bar=100nm.

Fig. 3.13b A vesicle (V) attached to the parental 'drippy gill' bacterium (DG). The vesicle membrane is apparently continuous with the bacterial outer membrane. Bar=20nm.

Fig. 3.13c A bacterial vesicle between the bacterium (DG) and the *Agaricus* wall (FW). The vesicle (V) detaches from the parent bacterium. Bar=50nm.

Fig. 3.13d A bacterial vesicle between the bacterium (DG) and the *Agaricus* wall. The vesicle (V) migrates through the intercellular space to the *Agaricus* hyphal wall (FW). Bar=50nm.

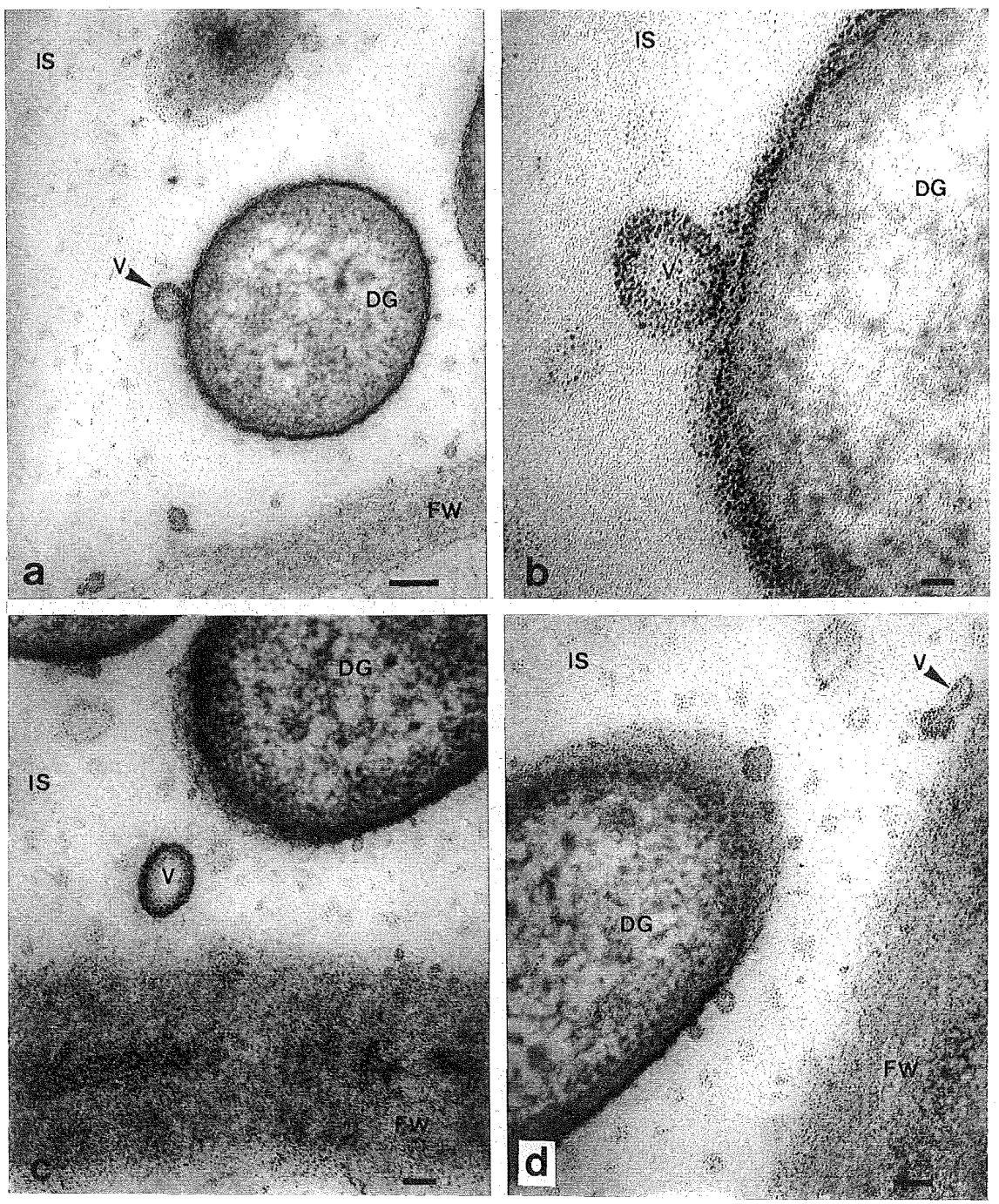

IS
V
DG
FW
a
IS
DG
V
b
DG
IS
V
FW
c
IS
V
DG
FW
d

FIGURE 3.14

Fig. 3.14 *Agaricus* wall-derived microfibrils. These are continuous with the outer fungal wall (FW), test positive for polysaccharide and are probably glucan microfibrils. IS-intercellular space, GM-glucan microfibril. Bar = 50nm.

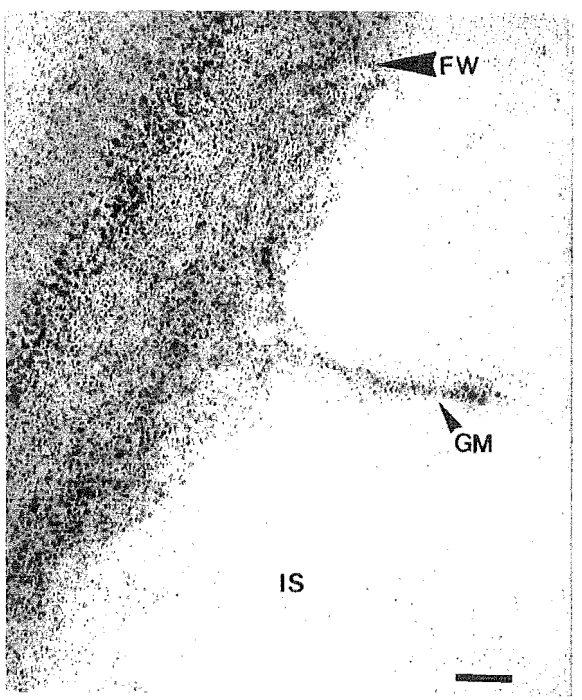

FW
GM
IS

FIGURE 3.15

Fig. 3.15a A negatively stained 'drippy gill' bacterium. This preparation shows that it is multi-flagellate. Bar = 100nm.

Fig. 3.15b 'Drippy gill' bacterium showing short appendages arising from the outer envelope. Bar = 50nm.

Fig. 3.15c A 'drippy gill' bacterium subjected to TCH staining. The short appendages shown here react indefinitely to TCH staining, suggesting they are composed of something other than polysaccharide. Bar = 100nm.

Fig. 3.15d A 'drippy gill' bacterium possessing short, peg-like appendages protruding from the outer bacterial envelope. Bar = 50nm.

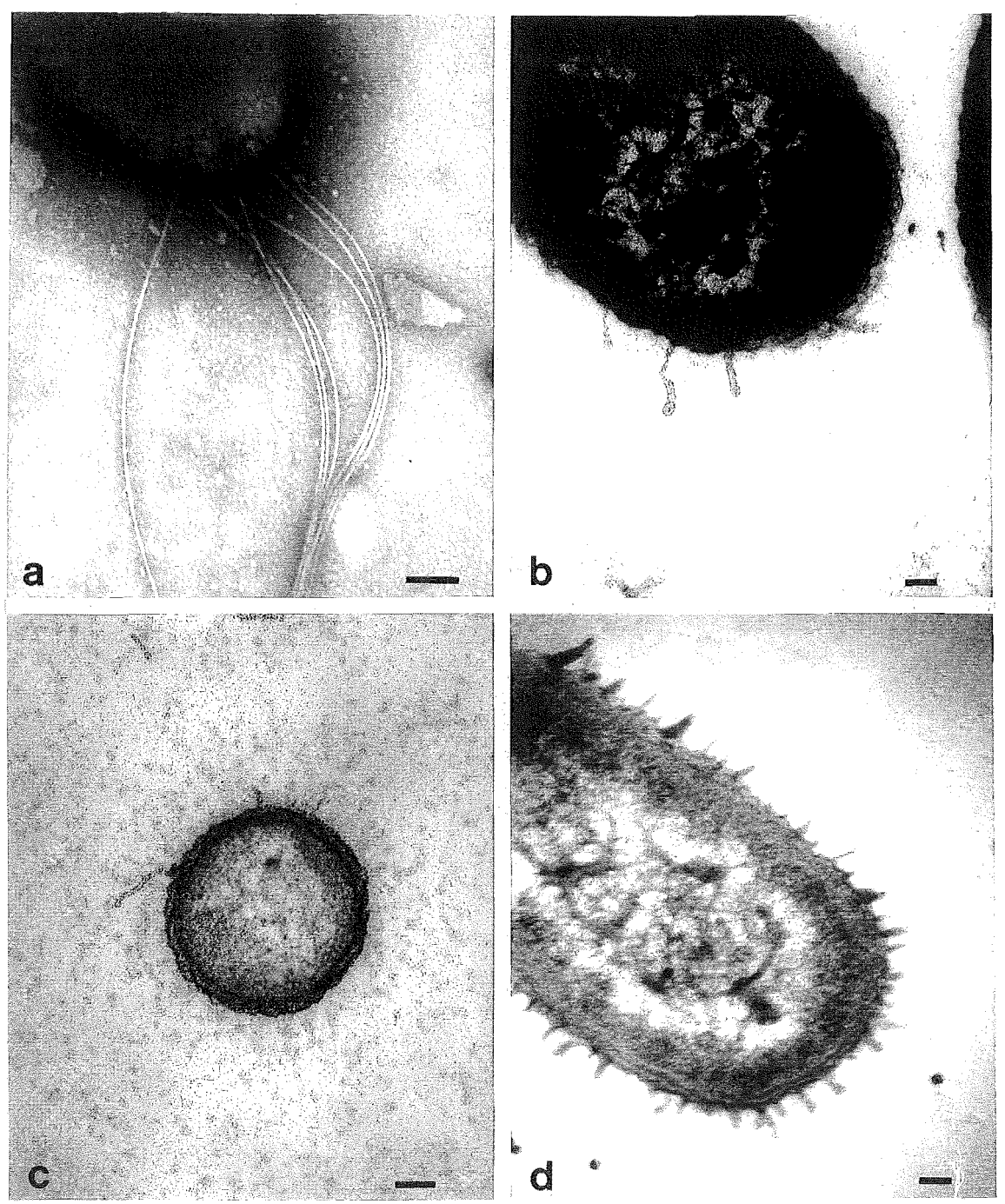

a
b
c
d

FIGURE 3.16

Fig. 3.16a A ruthenium red preparation of *P. agarici*. This preparation shows aggregations of polysaccharide (P) surrounding the bacterium. Bar = 100nm.

Fig. 3.16b A ruthenium red preparation of PV29 revealing aggregations of polysaccharide (P) around its outer membrane similar to *P. agarici*. Bar = 100nm.

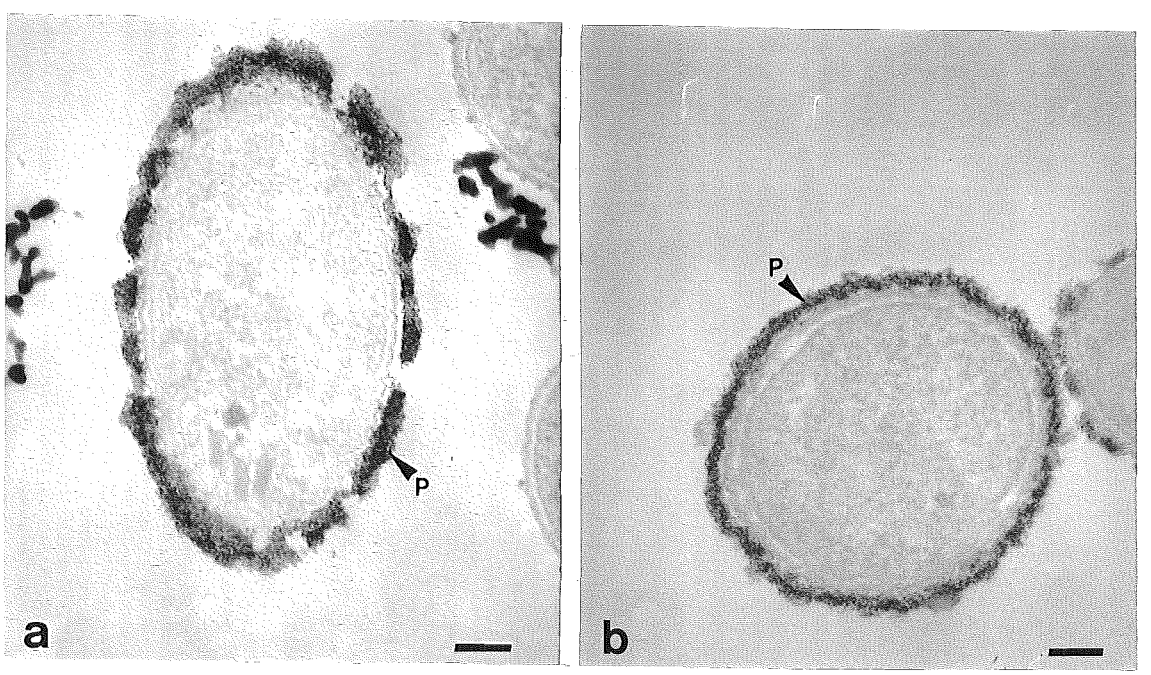

P
a
P
b

FIGURE 3.17

ES = extracellular space, FC = fungal cytoplasm
FW = fungal wall

Fig. 3.17a *Agaricus* vegetative mycelium subjected to wheat germ agglutinin-colloidal gold labelling. Negative enzyme control. Note the damage to the fungal wall and the paucity of gold label. FV-fungal vacuole. Bar = 100nm.

Fig. 3.17b *Agaricus* vegetative mycelium subjected to TCH staining. Negative enzyme control. Note the separation of the plasma membrane (PM) from the wall and also the diffusion of wall material in places (arrow). Bar = 100nm.

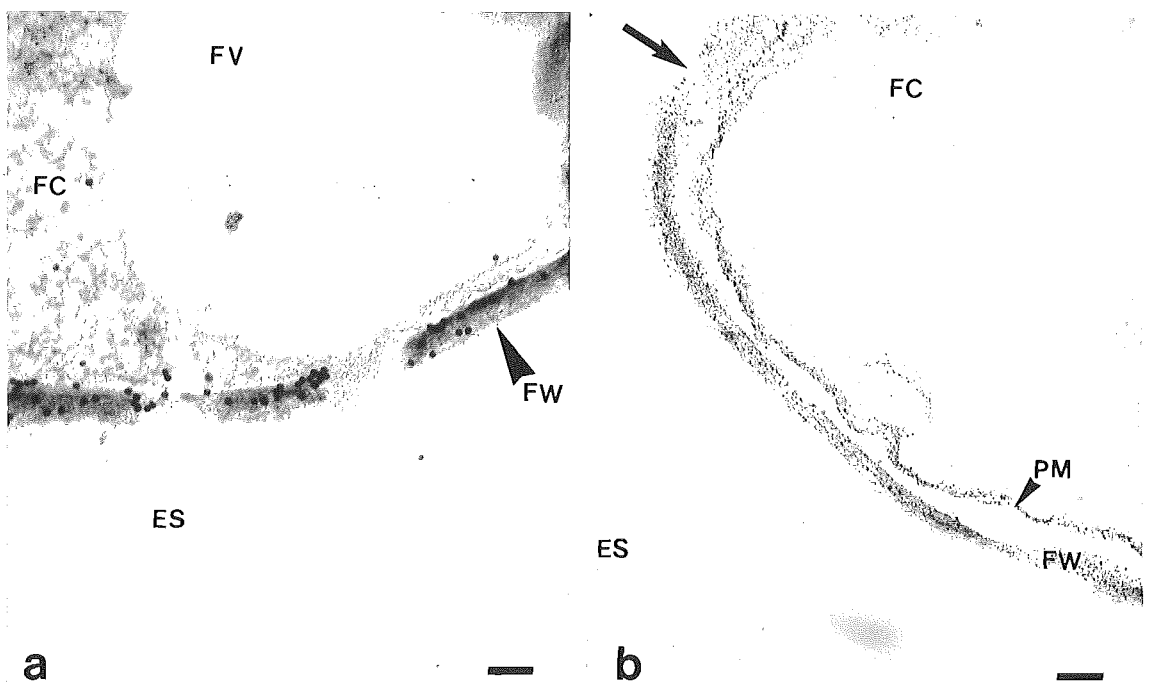

FV
FC
FW
ES
a
FC
PM
FW
ES
b

FIGURE 3.18

FC = fungal cytoplasm, FW = fungal wall
IS = intercellular space

Fig. 3.18a *Agaricus* vegetative mycelium treated with ß-glucanase followed by gold labelling. The chitin layer remains unaffected by enzyme treatment. There is evidence of a disturbance to the outer glucan layer (arrow). Bar = 100nm.

Fig. 3.18b *Agaricus* vegetative mycelium treated with ß-glucanase followed by gold labelling. Chitin (indicated by particulate gold), appears to be confined to a narrow band of hyphal wall. Bar = 100nm.

Fig. 3.18c Microfibrils released from *Agaricus* vegetative mycelium walls following treatment with ß-glucanase. Failure of gold particles to bind to the microfibrils (GM) indicates they are not composed of chitin. Bar = 100nm.

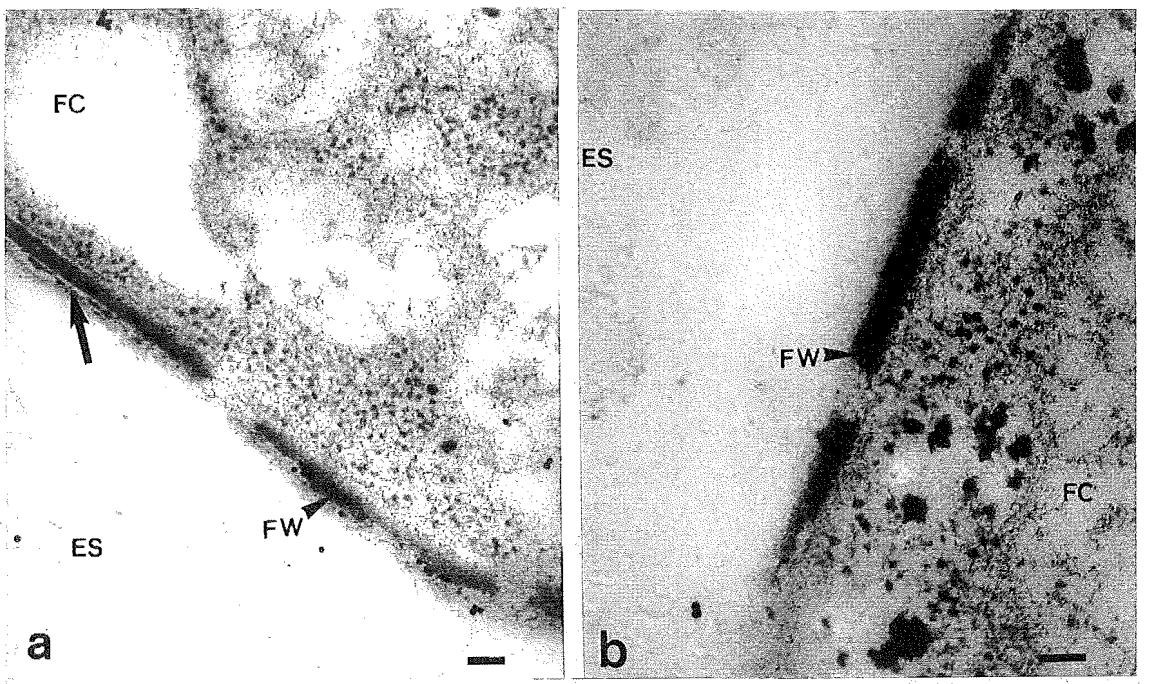

FC
ES
FW
a
ES
FW
FC
b
FC
FW
ES
GM
c

FIGURE 3.19

ES = extracellular space, FC = fungal cytoplasm

Fig. 3.19a *Agaricus* vegetative mycelium following ß-glucanase treatment. TCH staining shows that microfibrils are released from the fungal cell wall and liberated into the extracellular space. Bar=500nm.

Fig. 3.19b Wall breaches in ß-glucanase treated *Agaricus* vegetative mycelium. TCH staining demonstrates a gradual decrease of polysaccharide content of the exposed ends (arrow), as expected from an enzymatic degradation of wall material. Note the dark aggregations in the fungal cell. Bar=100nm.

Fig. 3.19c Microfibrils released from the ß-glucanase treated *Agaricus* hyphal wall. The microfibrils, derived from the outer glucan layers under the influence of ß-glucanase, stain positive for polysaccharides. Note the loss of integrity of the underlying fungal plasma membrane (PM). This appears similar to the plasma membrane of the TCH control section. Bar=100nm.

Fig. 3.19d Microfibrils released from the walls of ß-glucanase treated *Agaricus* vegetative mycelium. They appear to be continuous with the outer fungal wall glucan layers which suggests the microfibrils are composed of glucan. Bar=50nm.

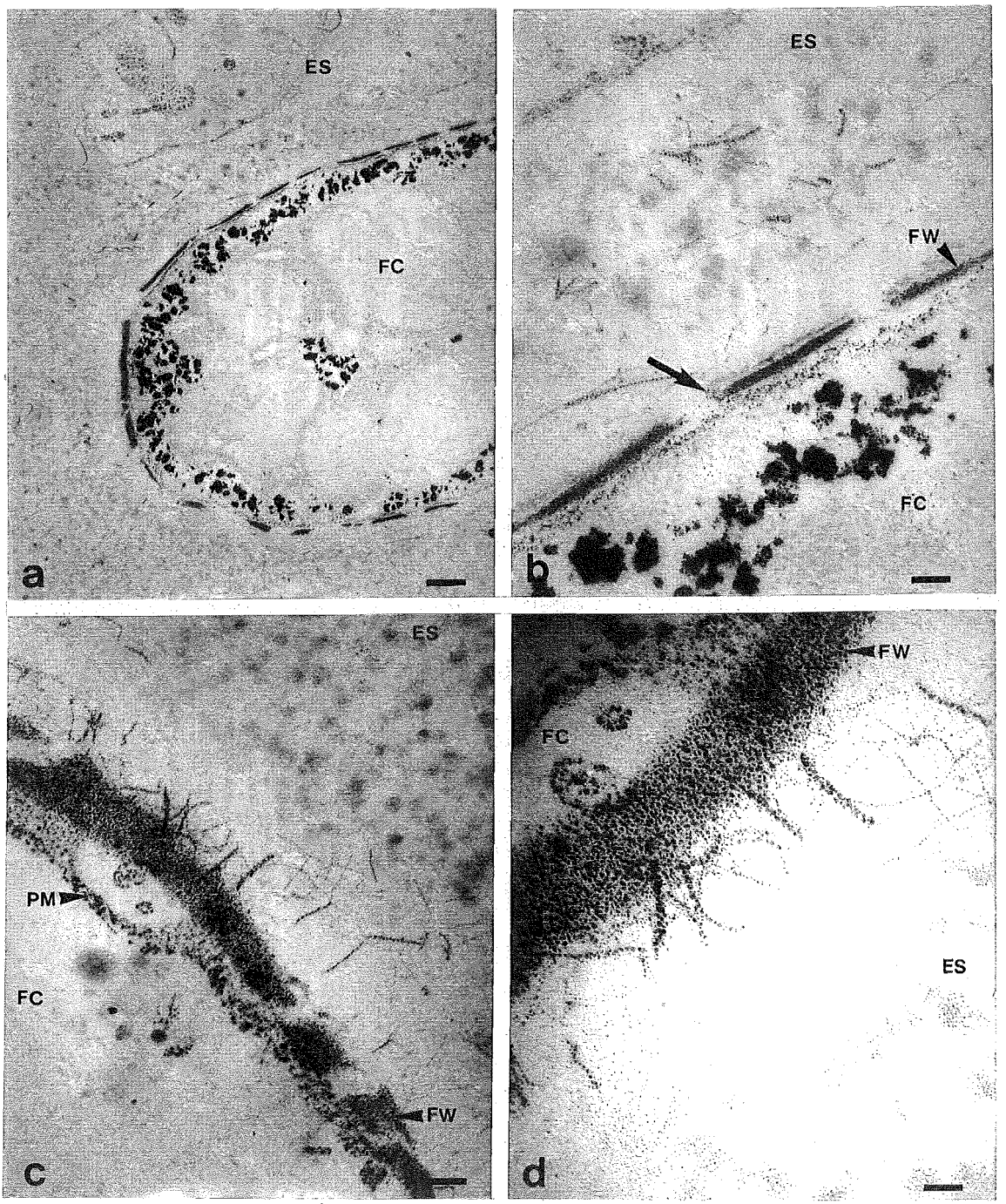

ES
FC
a
ES
FW
FC
b
ES
PM
FC
FW
C
FW
FC
ES
d

FIGURE 3.20

ES = extracellular space, FC = fungal cytoplasm
FW = fungal wall

Fig. 3.20a *Agaricus* vegetative mycelium following chitinase treatment. Gold labelling shows the chitin layer to be largely unaffected. There appears to be some loss of wall integrity. Bar = 100nm.

Fig. 3.20b *Agaricus* vegetative mycelium following chitinase treatment and subsequent gold labelling. There appears to be a disruption to the outer wall layers, but the chitin remains relatively unaffected as indicated by the gold particles binding to the inside wall layer. Bar = 100nm.

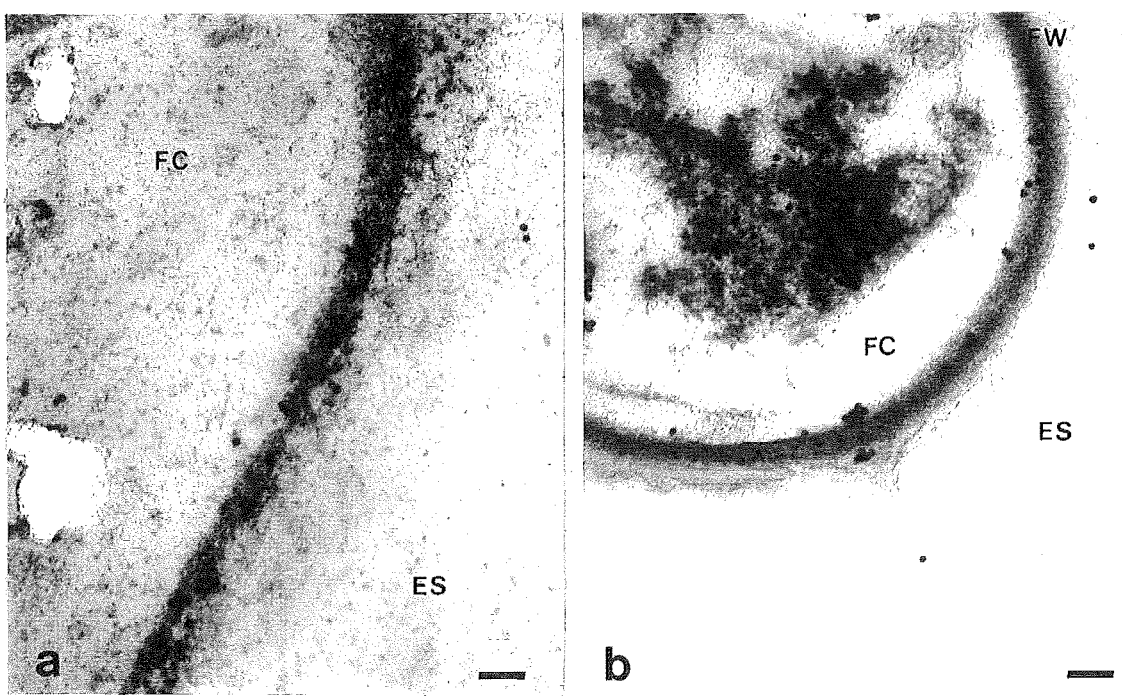

FC
ES
a
FW
FC
ES
b

FIGURE 3.21

ES = extracellular space, FC = fungal cytoplasm
FW = fungal wall

Fig. 3.21a *Agaricus* vegetative mycelium following chitinase treatment and TCH staining. Wall polysaccharides appear to be relatively intact following chitinase treatment, though a shadow seems to be present outside the wall (arrow) which may indicate a disturbance of material. Bar = 100nm.

Fig. 3.21b *Agaricus* vegetative mycelium following chitinase treatment and TCH staining. The breaches in the wall are apparent. Note the absence of fungal plasma membrane within the fungal cell. Bar = 100nm.

Fig 3.21c *Agaricus* vegetative mycelium following chitinase treatment and TCH staining. Abnormalities in the wall polysaccharide component occur frequently, showing aggregations of glucans, breaks and scattered wall material. However, the inner wall layers appear intact. Bar = 100nm.

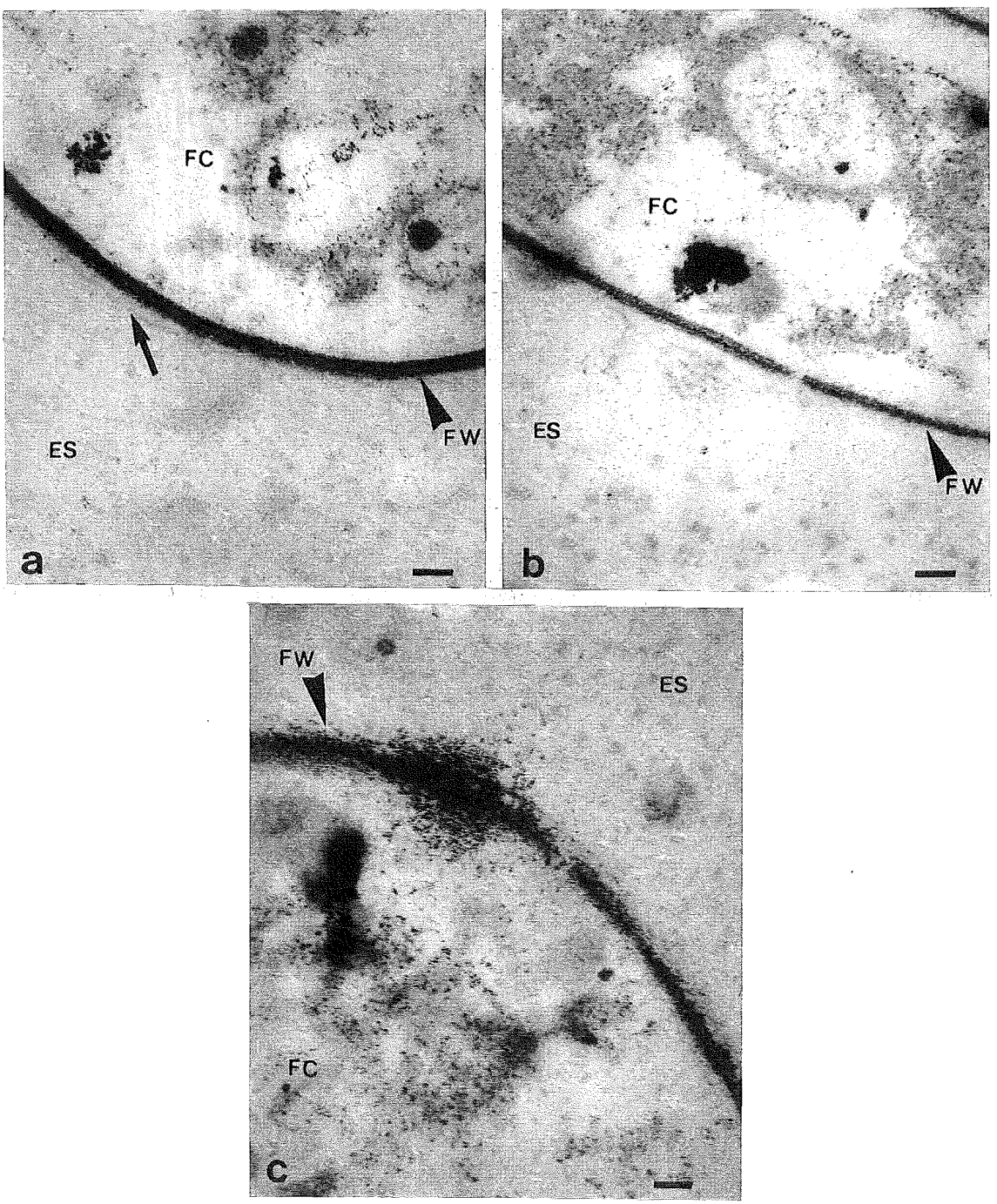

FC
ES
FW
a
FC
ES
FW
b
FW
ES
FC
c

FIGURE 3.22

ES = extracellular space, FC = fungal cytoplasm
FW = fungal wall

Fig. 3.22a *Agaricus* vegetative mycelium following ß-glucanase/chitinase treatment and subsequent gold labelling. Gold particles are confined to a scattered background intensity due to the hydrolysis of fungal wall chitin. Fungal walls appear as traces only. Bar = 100nm.

Fig. 3.22b *Agaricus* vegetative mycelium following ß-glucanase/chitinase treatment and subsequent gold labelling. Even when walls are detectable, there still is no gold binding to them, indicating they are devoid of chitin. Bar = 100nm.

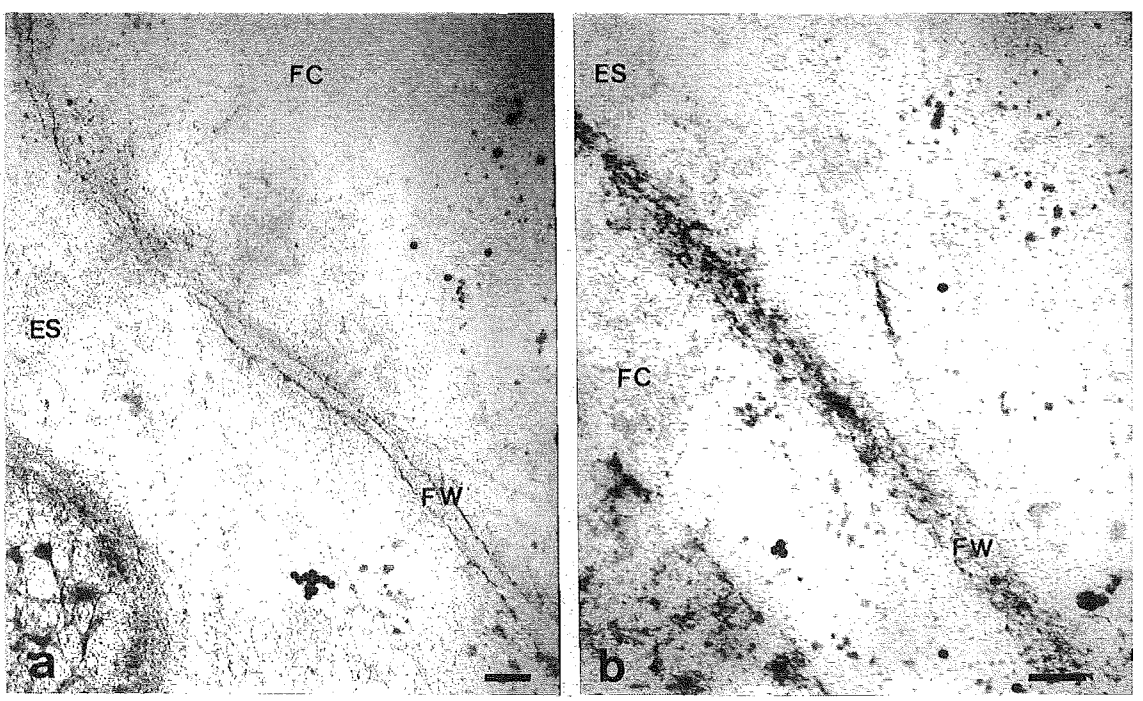

FC
ES
FW
a
ES
FC
FW
b

FIGURE 3.23

ES = extracellular space, FC = fungal cytoplasm
FW = fungal wall

Fig. 3.23a *Agaricus* vegetative mycelium following ß-glucanase/chitinase treatment and subsequent TCH staining. This treatment shows that the glucans are reduced and large segments of wall have been digested leaving large discontinuities. Bar=200nm.

Fig. 3.23b *Agaricus* vegetative mycelium following ß-glucanase/chitinase treatment and subsequent TCH staining. Wall layers are penetrated separately and breaches in successive walls are not adjacent. No plasma membranes are evident. Bar=100nm.

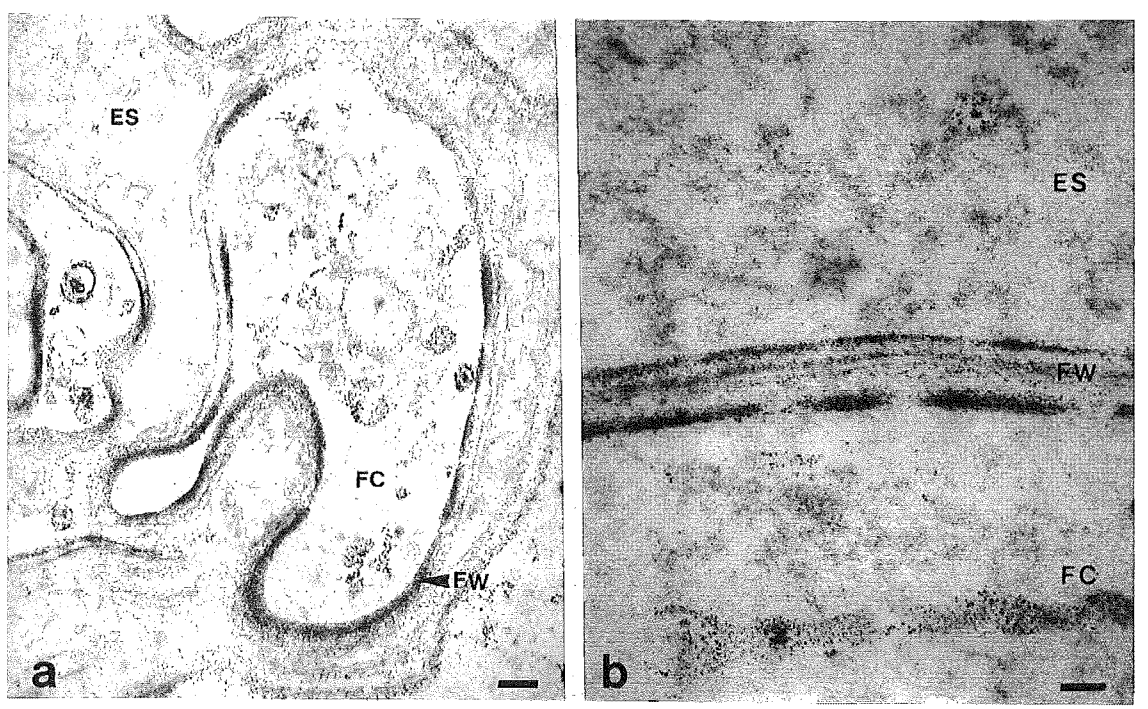
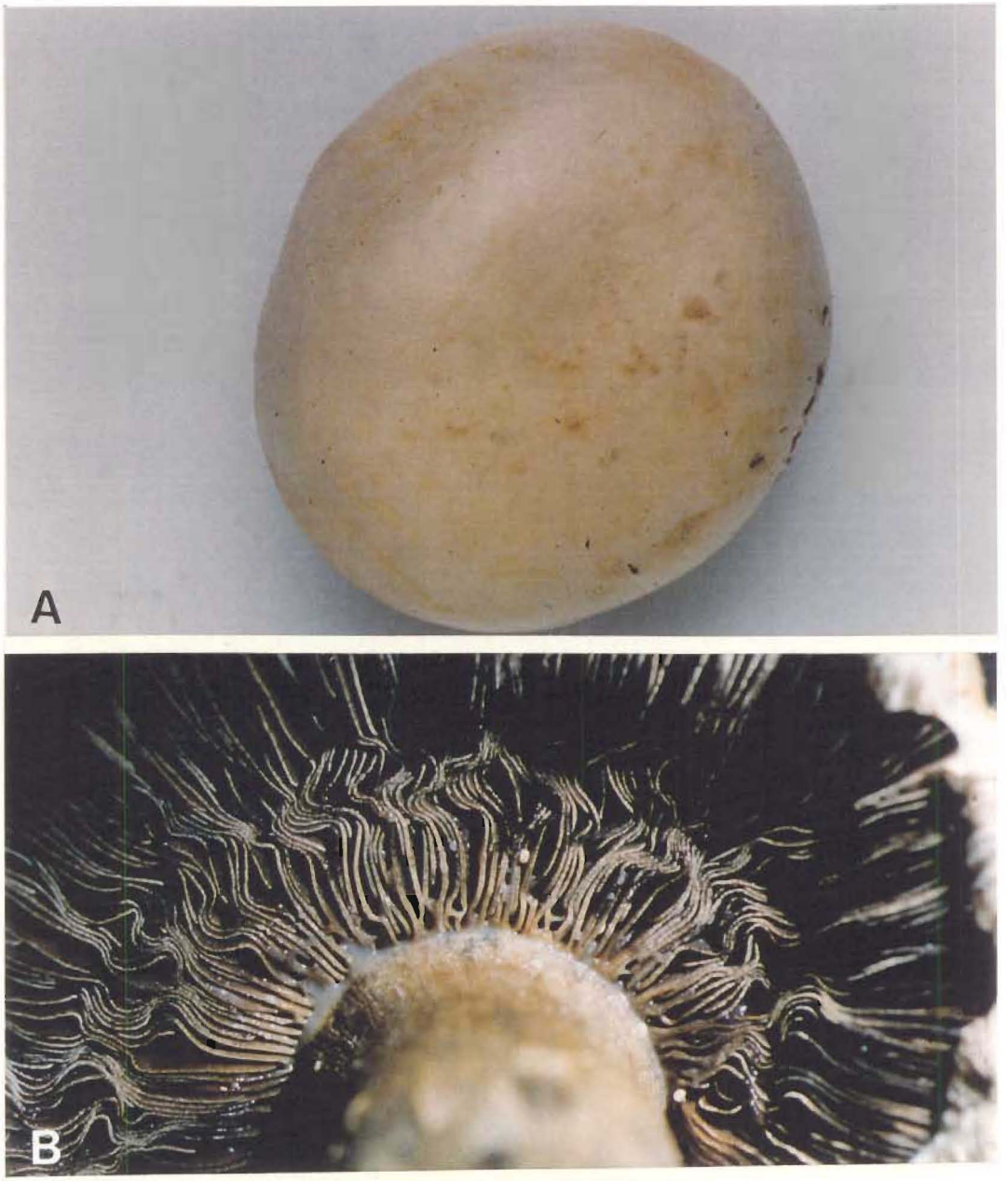

ES
FC
F-W
a
ES
F-W
FC
b

FIGURE 3.24

Fig. 3.24a *Agaricus* sporocarp inoculated with *Serratia liquefaciens*. Inoculation with this chitinase producer does not result in any obvious symptom.

Fig. 3.24b *Agaricus* sporocarp inoculated with *P. agarici*. Inoculation with this presumed ß-glucanase producer induces 'drippy gill' symptomology.

Fig. 3.24c *Agaricus* sporocarp inoculated with PMS164. This producer of both ß-glucanase and chitinase degrades the mushroom tissue.


A
B
C

3.4 DISCUSSION

The effects of both 'drippy gill' organisms, *P. agarici* and PV29, on *Agaricus* sporocarps are identical and the deleterious influences exerted on *Agaricus* hyphal walls and the extracellular matrix are independent of tissue type.

Degradation of the hyphal extracellular polysaccharidic matrix occurs in the immediate vicinity of the bacterial cells, but pockets remote from the influences of the bacteria retain their integrity. In some situations, isolated areas of the extracellular matrix appear to be degraded in the absence of bacteria, while the matrix as a whole remains intact. The presence of bacteria within close proximity is indicated, however, by remnants of bacterial flagella. Disturbances in the hyphal extracellular matrix are not confined to 'drippy gill' disease. Nott (1989), investigating ginger blotch and Skellerup (1984) working on brown blotch of *Agaricus* sporocarps caused by *P. gingeri* and *P. tolaasii* respectively, also observed disruptions to the hyphal extracellular matrix removed from the causal organisms. The disturbances seen in these cases may be the result of hyphal contraction and distortion due to the bacteria eliciting an adverse reaction from the mushroom, or they may be a hyphal reaction to bacterial toxins, for example tolaasin, in the case of *P. tolaasii* (Brodey *et al* 1991). These two blotch disorders are comparatively superficial and there is no major invasion of the sporocarp, certainly not on the scale evident in the case of 'drippy gill'. Widespread damage to hyphal cells, reported by Nott (1989) to consist of separation of wall layers, the loss of central vacuoles, damage to the dolipore parenthesome septum and in some cases total collapse and destruction of the hyphae, is not seen in the 'drippy gill' syndrome. Cole and Skellerup (1986) reported the invasion of mushroom cap hyphae by *P. tolaasii*, however, the ability of the brown blotch organism to actively gain entry to hyphal cells was not discussed. Considering the similarities between the two blotch organisms, it is possible that the intrahyphal bacteria observed by Cole and Skellerup (1986) gained entry via the broken, disrupted cells described by Nott (1989). The appearance of intrahyphal bacteria within disorganized and disrupted cells is not a new observation. Kalbarczyk (1987) noted the association between intrahyphal bacteria and the disorganization and in some cases, death, of the host

hyphal cell in mummy diseased *Agaricus* sporocarps. Those hyphal cells seen to be completely filled with bacteria were characterized by walls which were "vividly damaged", while those hyphae containing few bacteria showed cytoplasmic shrinkage around the bacterial cells. He noted the consistent appearance of the intrahyphal bacteria near wall damage, which led him to theorise the mode of entry as via the damaged hyphal wall. Again, whether the bacteria actively entered the hyphae, or exploited existing damage was not discussed. This is also the case in other reports of mummy disease. Intracellular bacteria were observed, but no method of entry discussed (Schisler *et al* 1968; van Zaayen and Waterreus 1974).

The symptoms of 'drippy gill' suggest the bacteria are intrahyphal in nature. Therefore, access to the interior of the hyphae is necessary and enzymes to enable penetration and to cause breakage of the walls would be a requisite. Petri dish assays have demonstrated that 'drippy gill' isolates are unable to hydrolyse chitin suspended in an agar medium, despite the inclusion of mushroom metabolites. It is not surprising, then, that wheat germ agglutinin-colloidal gold labelling indicates the inability of the bacteria to degrade the chitin content of hyphal walls. At no stage was the removal of chitin from 'drippy gill' sporocarp tissue indicated. Analysis of gold particle density reveals no variation in chitin content between hyphal walls exposed to bacteria and those which are remote from bacterial influence. From these two observations, it appears that neither *P. agarici* nor PV29 possess the ability to produce chitinase and therefore it is not a significant factor in the 'drippy gill' syndrome.

Membrane bound vesicles, originating from the bacterial outer envelope, are seen to migrate to the fungal wall through the intercellular space and may deliver enzymes responsible for the disruption of the outer glucan layers and release of polysaccharide microfibrils. Species of *Bacteroides* have been shown to produce vesicles from their outer membranes (Handley and Tipler 1986). Such vesicles were thought to contain lipopolysaccharides which assist in adhesion to oral surfaces (Slots and Genco 1984 in Handley and Tipler 1986). Published diameters of glucan microfibrils show they are of a range from approximately 10-14nm for α-glucan and 7-10nm for ß-glucan (Ruiz Herrera 1991). The large wall-derived microfibrils, having a diameter of approximately 13nm, would fall

within the limits of α-(1,3)-glucan. This is consistent with the observations of Garcia Mendoza *et al* (1987) who demonstrated α-(1,3)-glucan microfibril release from *Agaricus* vegetative hyphal walls following the removal of the overlying polysaccharide mucilage. The outer polysaccharide mucilage corresponds to the extensive ß-glucan extracellular matrix evident in mature sporocarps (Novaes-Ledieu *et al* 1987). The degradation of the extracellular matrix by 'drippy gill' bacteria results in the removal of the overlying wall mucilage and eventual release of α-glucan microfibrils. This is seemingly the case with the two mushroom blotch organisms. Nott (1989) observed disruption to the outer hyphal wall layers and associated this with the degradation of the hyphal extracellular matrix, suggesting that these effects were both due to a ß-glucanase enzyme. Skellerup (1984) came to a similar conclusion, though she described the extracellular matrix as an α-glucan. Lending support to this proposal is the observation that the 'pure' ß-glucanase treatment released microfibrils from hyphal walls in a similar manner. The vesicles, released by 'drippy gill' bacteria, may assist in the removal of the outer polysaccharide and indeed the extracellular matrix and thus release the underlying α-glucan microfibrils as observed.

It is widely accepted that organisms purporting to lyse fungal cells must possess both ß-glucanase and chitinase which work sequentially to digest firstly the outer glucan and then the inner chitin (Potgieter and Alexander 1966; Michalenko *et al* 1976), which is embedded in a ß-glucan matrix (Novaes-Ledieu *et al* 1987). The fact that the two components of the ß-glucan/chitin matrix are inextricably covalently linked (Sietsma and Wessels 1977, 1979; Mol *et al* 1988) confers a degree of resistance against exogenous hydrolytic enzymes (Mahadevan and Tatum 1967). The single enzyme treatments of *Agaricus* vegetative mycelium in this study confirm this. Individually, neither chitinase nor ß-glucanase was able to lyse hyphal cells. However, in unison, these enzymes were able to digest large tracts of *Agaricus* hyphal wall. 'Drippy gill' bacteria are unable to produce chitinase, therefore their ability to actively penetrate hyphal walls is in doubt.

It has been shown by inference only that 'drippy gill' bacteria produce ß-glucanase. The failure to demonstrate the hydrolysis of both pure glucan

substrates and isolated *Agaricus* wall fractions is disconcerting. ß-glucanases are semi-constitutive and inducible (Reese and Mandels 1959) and are ubiquitous enzymes, common in both fungi and bacteria. The assay methods employed would detect the products of both the exo- (glucose and short oligosaccharides from end-wise chain cleavage) and the endo- (longer oligosaccharides from random chain cleavage) type activities (Bull and Chesters 1966). However, according to Novaes-Ledieu *et al* (1987), the predominant linkage in the *Agaricus* hyphal extracellular mucilage is ß-(1,4), a linkage type not tested among the pure glucan substrates due to its unavailability. Subsequent tests wih lichenin, a ß-(1,3)/(1,4)-glucan, showed that it was not hyrolysed by the 'drippy gill' isolates. A substrate specific or linkage specific ß-glucanase may not have been induced by the substrates tested. Glucanase activity was not detected in the crude *Agaricus* wall fractions perhaps because the fungal wall isolation procedures require severe chemical treatments such as exposure to hot alkali for a number of hours. The ability of a glucose polymer to tolerate and withstand such treatment unaltered is questionable (Rosenberger 1976; Avellan *et al* 1986).

Fungal wall discontinuities are prevalent throughout 'drippy gill' affected sporocarps. 'Drippy gill' bacteria do not produce chitinase and are therefore unlikely to enzymatically penetrate the hyphal wall. Close investigation of the wall breaks, in association with colloidal gold labelling, show these breaks to be mechanical in nature. As 'drippy gill' bacteria remove the hyphal extracellular matrix, they also remove the lubrication which helps hyphae slide past each other during processes such as stipe elongation. Exposure to unusual frictional forces may induce areas of weakness within the wall which manifest later as breaks. The damage, However, is not as marked or severe as that described by Nott (1989) and Skellerup (1984). Whatever the cause of the break, it appears that 'drippy gill' bacteria exploit pre-existing wall discontinuities in order to gain entry to *Agaricus* hyphae in much the same way as envisaged by Rainey (1989) in the case of *P. putida*.

'Drippy gill' bacteria interact and disrupt the outer hyphal wall layers. It has been demonstrated that the degree of adhesion is similar to *P. tolaasii* (Rainey 1989), the brown blotch organism, yet there is little physical

evidence for this at the ultrastructural level. They possess short peg-like appendages which would no doubt be useful for adhesion, a crucial facet of pathogenesis and colonization (Kolenbrander 1991). Considering the fact that they do not actively penetrate the hyphal wall and they are seen to pass through the sporocarp, it is difficult to comprehend the advantage of bacterial adhesion to the hyphae. However, Rainey (1989) observed a similar phenomenon with the saprophytic *P. putida*.

It is interesting to note that *Serratia liquefaciens* produces chitinase and does not incite any visible symptom on *Agaricus* sporocarps (Fig. 3.24a). *P. agarici* produces ß-glucanase and causes the symptoms of 'drippy gill' (Fig. 3.24b). However, PMS164 produces both chitinase and ß-(1,3)-glucanase and degrades the cap tissues (Fig 3.24c). The two individual enzymes working separately are unable to degrade the fungal hyphal wall, but while working in unison are able to totally hydrolyse the fungal tissue to a liquid consistency, an observation consistent with that of Michalenko *et al* (1976).

'Drippy gill' organisms possess a particular physiological function, the ability to produce an agent, possibly a ß-glucanase enzyme, which allows them to invade and colonize the intercellular spaces of *Agaricus* sporocarps. Other mushroom pathogens tend to be more superficial such as blotchers (*P. gingeri* and *P. tolaasii*) or more damaging such as the soft rots, in particular *P. gladiolii* pv *agaricicola* (PMS164), which act by degrading all tissue. *P. tolaasii* has, nonetheless, been isolated from 'drippy gill' affected hymenial tissues of *Agaricus* sporocarps, probably by exploiting the interhyphal channels created through the pileus and 'piggybacking' from the cap surface through to the hymenium with *P. agarici*, assisted by the influence of gravity and watering. That 'drippy gill' bacteria are unable to produce the chitinase enzyme ensures that they are unable to actively penetrate fungal hyphae. The intrahyphal occurrence of 'drippy gill' is, however, irrefutable, but it is a facultative process rather than an obligate one.

CHAPTER FOUR

CONCLUDING DISCUSSION

4.1 CONCLUDING DISCUSSION

4.1.1 General Considerations

`Drippy gill' disease of cultivated *Agaricus* mushrooms is caused by *P. agarici*, a fluorescent pseudomonad (Young 1970). The causal organism of a similar ooze disease, PV29, purported to be distinct from *P. agarici* (Rainey and Cole 1988), was shown to be indistinguishable from *P. agarici* and was considered to be the same species. The widely used LOPAT classification system for phytopathogenic fluorescent pseudomonads (Lelliott *et al* 1966) was found wanting with respect to allocating *P. agarici* to a distinct group consistent with its LOPAT attributes. Difficulties in allocating *P. tolaasii* to a LOPAT group were encountered by Lelliott *et al* (1966). To account for this, they regarded the brown blotch organism as a saprophyte, due to its prevalence in mushroom culture compared with the relatively low degree of disease expression. They argued that only when *P. tolaasii* becomes virulent, responding to certain environmental triggers, does a toxic metabolite incite blotching.

The reverse transformation from the pathogenic to the saprophytic state is most commonly seen with *P. tolaasii* and *P. gingeri* as a change in colony morphology from smooth to rough in plate culture. *P. agarici* does not readily undergo this transformation. This raises interesting questions as to the exact relationship between the `saprophytic' and the `pathogenic' bacterial flora of the mushroom bed. Rainey (1989) noted that the smooth colonial form of the saprophytic *P. putida* possessed the ability, given the right conditions, to adhere to mushroom mycelium at a similar rate to *P. agarici.* Similarly, he observed the `end on' attachment of this same bacterium to mycelium and also the facultative intrahyphal occurrence, entry having been gained (he assumed) through a mechanical break. These observations are very similar to those made of *P. agarici* in this study. The relationships between the bacteria of the casing layer microflora appear to be complex, requiring further clarification.

Mushroom pathogens present problems when attempting to classify and group them using a system such as LOPAT based on so few characters.

In addition the ability of these organisms to transform with consequent changed physiology compounds the problem. That *P. tolaasii* and *P. agarici* have both been relegated to the `dumping ground' of the pseudomonads (Palleroni 1984) reflects the difficulty these pathogens present in attempting a classification.

`Drippy gill' disease is characterized by a profuse bacterial ooze emanating in droplets from hymenial lamellae and longitudinal splitting of the stipe with an associated bacterial ooze. The nature of symptom expression has led people to believe the causal organism may exist and indeed be transmitted intrahyphally. Evidence to the contrary has been presented in this study. It has been demonstrated that protective membranes such as the pileipellis offer little protection against the `drippy gill' causal organisms, thus highlighting the susceptibility of embryonic sporocarps to invasion. That the symptoms are confined to the hymenium and stipe is correct (Young 1970; Rainey and Cole 1988). However, bacteria are present throughout all sporocarp tissues with the exception, apparently, of the inner stipe core tissues. It is the location of the hymenium and stipe with respect to the site of infection which facilitates symptom expression and not any predilection of the bacteria.

If the causal bacteria were to be transmitted intrahyphally, they would require ß-glucanase and chitinase to first penetrate the hyphal wall (Potgieter and Alexander 1966; Michalenko *et al* 1976), and then to dissolve the septum (Janszen and Wessels 1970) to facilitate transmission. It has been demonstrated that *P. agarici* does not produce chitinase, a feature which effectively precludes the `drippy gill' organism from actively entering *Agaricus* hyphal cells. This is consistent with observed intrahyphal bacteria entering disrupted, usually dead, hyphal cells through broken walls, in a similar manner to that described by Kalbarczyk (1987). However, evidence was obtained showing a bacterium apparently actively entering a hyphal cell. The nature of this observation remains obscure. Although chitinase is lacking, it is likely that ß-glucanase is produced by *P. agarici*.

The inability to produce a toxin active against *Agaricus* mycelium may allow `drippy gill' bacteria to infiltrate the sporocarp without having to negotiate a `plug' of modified hyphae produced as a reaction to the toxin

as seen in brown blotch and ginger blotch (Skellerup 1984 and Nott 1989 respectively).

P. agarici is a mushroom pathogen which differs from the others found in the mushroom casing layer. It does not produce a toxin, unlike *P. tolaasii* and *P. gingeri*; it is not a soft rotting organism like *P. gladioli* pv *agaricicola*, nor is it intrahyphal as is (supposedly) the causal organism of mummy disease. *P. agarici* appears to be very similar to the saprophytic *P. putida* in many ways, most interestingly in its ability to promote mycelial growth. Overall, *P. agarici* would seem to have saprophytic qualities inconsistent with its pathogenic behaviour.

4.1.2 Development of `Drippy Gill' Disease

From evidence gathered in this study, the following aetiology and epidemiology of `drippy gill' disease of cultivated *Agaricus* is proposed.

The causal organism, *P. agarici*, is introduced into the mushroom growing shed via the casing material. As the embryonic basidiomata develop and emerge through the casing layer they come into contact with the bacterium. Once in contact with the immature cap, the bacteria penetrate the protective membranes by degrading the extracellular matrix of the hyphae. As the bacteria do not produce a mycelial toxin the mushroom does not form a physical barrier to invasion as seen in ginger blotch and brown blotch. `Drippy gill' bacteria are able to continue degrading the extracellular matrix and, under the influence of gravity and the trickling effect of watering passing through the intercellular spaces, are seen to exit at the hymenium. The hymenium represents the `terminus' of the path through the pileus, hence the accumulation of bacteria on the hymenial lamellae. Fed by a continually increasing reservoir of bacteria from above, combined with the effects of watering and gravity, the bacterial droplets grow and eventually fall onto the mushroom bed. Those bacteria which enter the stipe continue degrading the adhesive hyphal extracellular matrix until the hyphae separate and the stipe splits, exposing the causal organism as an ooze coating the inner faces of the

splits. The ooze is then able to 'reinfect' the surrounding mushroom bed. If the bacteria were obligately intrahyphal, symptoms would perhaps be expressed on the pileus itself. The cap hyphae form an anastomosing network which would deliver the bacteria to the cap periphery where symptoms such as splitting would manifest. The fact that this is not the case and that hymenial droplets occur more or less beneath the point of infection on the cap suggest the bacteria are predominantly confined to the extracellular matrix.

Two methods considered valuable for the elucidation of the proposed disease development were investigated but found to be unsuitable.
The Lux gene was successfully introduced to the *P. agarici* genome which allowed the bacterium to be detected by the consequent luminescence. The intensity of luminescence was insufficient, however, to allow detection of the bacterium in anything other than large colonies, inappropriate for following bacteria within mushroom tissue.
Secondly, fluorescent dyes were used to stain various parts of the fungus and the bacteria in order to investigate their interaction. Fluorochromes were tested, DAPI (4,6 Diamidino-2-phenylindole) and Hoescht 33258 (2'-[4-Hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole), in an effort to stain the bacterial DNA. As the bacterial DNA is not confined within a membrane, these fluorochromes, in effect, stain the entire bacterial cell. A fluorescent dye, FITC (fluorescein isothiocyanate), was also used to stain *Agaricus* hyphal walls, looking for the presence of wall breaches. This was conjugated with both wheatgerm agglutinin and chitinase, molecules specific for the major fungal skeletal polysaccharide, chitin. As with the Lux system, these fluorescent procedures did not prove to be useful for this particular application.

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APPENDICES

APPENDIX A

'Cavity-cap' Disease of *Agaricus bitorquis* caused by *Pseudomonas cepacia*

INTRODUCTION

Recently, a commercial mushroom grower in Christchurch, New Zealand, reported disease symptoms on sporocarps from an *A. bitorquis* crop. Symptoms noted ranged from mild blotching to deep pitting, scaly cap and in severe cases, complete degradation of all sporocarp tissue including the stipe. On mature sporocarps, the symptoms appeared as characteristic large eroded areas with peripheral lesions often extending from the cap margin to the stipe where the complete degradation of all intermediate tissues and in due course, degeneration of the stipe is seen (Fig. 5.1). In many cases, the outer tissue of the sporocarp remained intact and apparently healthy, but closer examination revealed the cap to be hollow, the underlying tissues having been reduced to a brown, soupy consistency.

MATERIALS AND METHODS

The causal organism (designated CANU-PMS164 and deposited in the culture collection of the Department of Plant and Microbial Sciences, University of Canterbury) was isolated from diseased mushrooms by streaking a loopful of degraded tissue from a surface lesion directly onto KB agar and incubating at 25^{o}C in darkness for 48h. The dominant bacterial colony was purified by streaking out onto KB and incubating for 48h. The bacterium was maintained on KB by subculturing every 72h. For longterm storage, a single colony was transferred to 3ml nutrient broth and incubated in shaking culture at 25^{o}C for 24h. 0.8ml of this culture was

transferred to an Eppendorf tube containing 0.2ml sterile glycerol, mixed and placed immediately in a -80^{o}C freezer.

Tests to satisfy Koch's postulates and confirm causation were made by suspending pure colonies in 5ml of sterile distilled water to a density of approximately 10^8 cells/ml (Hawksley haemocytometer) and a loopful of suspension applied to the cap of each of 15 surface disinfested (1% sodium hypochlorite) excised *A. bitorquis* sporocarps in sterile petri dish lids. Controls were surface disinfested and inoculated with a loopful of sterile distilled water. Sporocarps were selected from both the button and fully open stages of development. These were placed in a sealed container lined with moist paper towels and incubated at 25^{o}C in darkness for 72h. The causal bacterium was re-isolated in pure culture from the resultant induced lesions.
The production of an antifungal metabolite was tested by plating *Agaricus* mycelium against a bacterial streak and a cell-free culture filtrate (2.2.5).

In order to identify the bacterium, the following tests were carried out:
Size and general morphology were determined by Gram's stain, flagellation by Ryu's stain (Kodaka *et al* 1982) and negative staining with 2% phosphotungstic acid and transmission electron microscope examination, KOH solubility (Suslow *et al* 1982), presence/absence of poly-ß-hydroxybutyrate inclusions, levan production, starch hydrolysis, gelatin liquefaction and 'Tween' 80 hydrolysis (Lelliott and Stead 1987), fluorescence on KA and KB media (King *et al* 1954), arginine dihydrolase (Thornley 1960), potato soft rot (Lelliott *et al* 1966), arginine, ornithine and lysine decarboxylases (Cowan 1974), H_2S from cysteine (Dye 1968), nitrate reduction (API Systems), oxidase (Kovacs 1956), tyrosine hydrolysis (King and Phillips 1985) and carbohydrate utilization (API 50CH strips using the medium of Goor *et al* 1986).

RESULTS

All sporocarps inoculated with pure cultures of the isolated bacterium showed a range of symptoms, from pitting through to hollowing out of the cap, identical to those exhibited by the original diseased mushrooms. Control sporocarps, surface disinfested and inoculated with a loopful of sterile distilled water, showed no such symptoms. Koch's postulates were satisfied by re-isolation of the bacterium from the diseased mushrooms. (Fig. 5.2)

The causal organism is a non-spore-forming rod-shaped bacterium (0.5-1.0 x 3.0-3.5μm), Gram negative, KOH soluble and motile by three polar flagellae. Poly-ß-hydroxybutyrate inclusions were identified within the cells. Fluorescence on KA and KB media, arginine dihydrolase, potato soft rot, levan production, arginine, ornithine and lysine decarboxylases, H_2S from cysteine and nitrate reduction were all negative. Oxidase, tyrosine hydrolysis, starch hydrolysis, gelatin liquefaction and 'Tween' 80 hydrolysis, were positive. On KB, the bacterium produced yellow, irregular colonies with entire, crenulate margins and a diffusable, non-fluorescent yellow pigment (Fig. 5.3a). Colonies reached 2-3mm in diameter after 72h incubation at 25^{o}C. The toxin assay indicated that the bacterium inhibits *Agaricus* vegetative mycelium (Fig. 5.3b), suggesting a compound active against the fungus is produced. There was no growth recorded on KB after 5d incubation at 4^{o}C and 42^{o}C, but when these plates were reincubated at 25^{o}C, growth was apparent after 36h.

The bacterium was able to utilize the following after 72h: glucose, D-xylose, D-ribose, L-rhamnose, adonitol, glycerol, D-arabinose, L-arabinose, galactose, fructose, mannose, dulcitol, inositol, mannitol, sorbitol, N-acetylglucosamine, aesculin, maltose, lactose, sucrose, trehalose, xylitol, L-fucose, D-arabitol, gluconate, 2-ketogluconate, 5-ketogluconate, caprate, adipate, malate, citrate, phenylacetate and urea.

Utilization of the following was negative after 72h: erythritol, L-xylose, α-methyl-D-xyloside, L-sorbose, α-methyl-D-glucoside, amygdalin, salicin, melibiose, inulin, D-melezitose, raffinose, amidon, glycogen, ß-gentibiose,

D-turanose, D-lyxose, D-tagatose, D-fucose, L-ornithine, D-tryptophan, and L-arginine.

From the above results, the causal bacterium was identified as *P. cepacia*. The ability of PMS164 to survive and multiply on the *P. cepacia* selective medium, PCAT of Burbage and Sassar (1982), supports this identification.

DISCUSSION

This is the first report of a non-fluorescent pseudomonad causing disease of mushrooms. A common and widespread soil organism, *P. cepacia* has been described earlier as the cause of sour skin of onions (Burkholder 1950) and bacterial brown spot of orchids (Tsuchiya *et al* 1986). Occurrence and distribution of *P. cepacia* is not confined solely to plant sources. It has also been isolated from clinical specimens of human origin, particularly cystic fibrosis sufferers (Goldmann and Klinger 1986) and also from equipment and solutions in hospital environments (Gilardi 1983), demonstrating the ability of this species to adapt to diverse habitats. *A. bitorquis* had been selected by the grower because of a recent history of viral disease affecting *A. bisporus* crops. *A. bitorquis* requires a higher temperature regime than *A. bisporus* and at this elevated temperature, the incidence of viral infections has markedly decreased due to the inability of the virus to survive. However, *P. cepacia* thrives in the increased temperature.
PMS164 has been shown to produce ß-glucanase (Chapter 3) and utilize N-acetylglucosamine (Appendix A). The fact that live bacteria are required to inhibit *Agaricus* mycelium suggests the active compound may be enzymatic.

PMS164 differs in some characteristics from the type strain of *P. cepacia*, ATCC25416 (Table 5.1). *P. cepacia* is a diverse species occupying a range of habitats and therefore these differences might be expected to occur, but are not sufficient to warrant a separate species designation.

The term 'hollow-cap' best descibes the symptoms of this disease however it is a term already used to describe a physiological disorder occasionally afflicting sporocarps. Thus the term 'cavity-disease' is suggested as a common name for this condition.

Concomitant with this disease occurrence, a similar condition was reported on mushrooms in the United Kingdom by Lincoln *et al* (1991) and the causal organism identified as *P. gladioli* pv *agaricicola*. Fatty acid analysis of the above *P. cepacia* showed an identical profile with *P. gladioli* pv *agaricicola* and a just prior publication by the United Kingdom group would indicate the 'cavity-cap' causal organism to be now designated *P. gladioli* pv *agaricicola*.

Table 5.1 Differentiating Characteristics of PMS164 and ATCC25416

CHARACTERISTIC	PMS164	ATCC25416
Starch Hydrolysis	+	-
Gelatin Liquefaction	+	-
Mushroom Blotch	+	-
Growth at 42°C	-	+
Pigment Colour on KB	yellow	green-yellow
Colony Surface	textured	smooth
UTILIZATION OF:		
D-Fucose	-	+
Lactose	+	-
Maltose	+	-
Salicin	-	+
Urea	+	-

+ denotes a positive result, - denotes a negative result

FIGURE 5.1

Fig. 5.1a *Agaricus bitorquis* sporocarp showing 'cavity cap' symptoms. Note the split, under-developed pileus and the dark brown coloured lesions.

Fig 5.1b *Agaricus bitorquis* sporocarp showing 'cavity cap' symptoms. Note the elongated stipe and pervasive cavity on the poorly developed pileus.

Fig 5.1c *Agaricus bitorquis* sporocarp showing 'cavity cap' symptoms. Note the stipe degeneration (sd), the pervasive peripheral cap lesions (cl) and hollowing out of the cap (h).


A
B
h
cl
sd
C

FIGURE 5.2

Agaricus bitorquis caps

Fig. 5.2a Control cap showing no symptoms of 'cavity' disease.

Fig 5.2b Deep pitting and hollowing out of cap.

Fig 5.2c 'Cavity' syndrome. Seemingly healthy area of outer tissue (arrow) adjacent to a deep pit.

Fig 5.2d The same cap as in Fig. 5.2c seen in longitudinal section, showing intact outer tissue (arrowed) with the underlying tissues reduced to a soupy consistency. The stipe has become detached as a result of the degradation of tissues at the point of attachment to the cap (s).

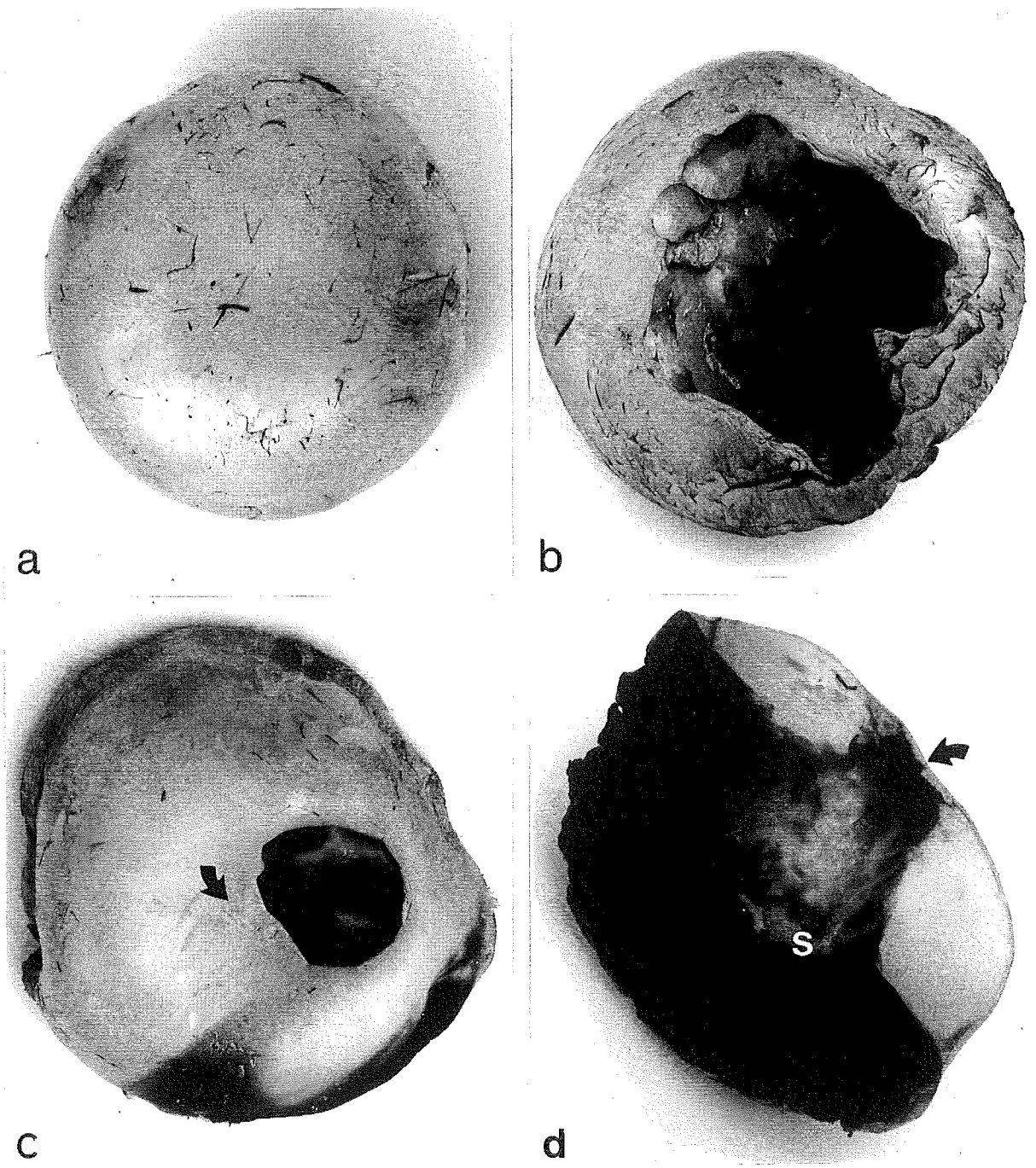
a
b
c
d
S

FIGURE 5.3

Fig. 5.3a PMS164 streaked on to KB agar. Note the crenulate colony margins, the wrinkled surface texture and diffusible pigment.

Fig 5.3b PMS164 toxin assay. The bacterium has been plated against *Agaricus bisporus* mycelium on a CMM agar plate. Note the inhibition around the streak, yet no obvious effect exerted by the cell-free culture filtrate on the assay disc.


A


B

APPENDIX B

A bacterium isolated from chitin-amended soil was shown to produce haloes of clearing on chitin agar (3.2.1.2) and was used as a positive control organism for testing chitinase production.

To identify the bacterium, a gram stain was initially completed and the reaction and morphology noted. As a result of these initial tests, both a 20E and 10S API strip (API Systems) was inoculated, incubated and read. The unknown bacterium was identified from the strips as *Serratia liquefaciens*. To confirm this, a number of nutritional and biochemical tests were completed and the results compared with the characteristics of *S. liquefaciens* as listed in Bergey's Manual of Systematic Bacteriology Volume 1 (2nd Edition 1984).

The following tests were carried out in addition to those on API strips: 'Tween' 80 hydrolysis (Lelliot and Stead 1987); L-arginine decarboxylase (Cowan 1974); growth at 4, 25, 30, 37 and 42^{o}C; growth in 7, 8, 9 and 10% saline; acid from meso-inositol, salicin, adonitol, L-arabinose, D-xylose, lactose, D-melezitose, D-sorbitol, and raffinose (Klement *et al* 1990); utilization of ß-alanine, DL-arginine, D-cellobiose, glycerol, inulin, D-lactose, L-ornithine, sarcosine, L-tryptophan, DL-ß-phenylalanine, 95% ethanol (Klement *et al* 1990); utilization of acetate, hippurate, benzoate, propionate and tartrate (Dye 1968).

The tests all yielded identical results for both the unknown and *S. liquefaciens* except the following: utilization of DL-arginine, D-cellobiose, hippurate and tartrate.

From these test results, it was concluded that the unknown positive chitinase producer is *S. liquefaciens*.

APPENDIX C

Raw data and ANOVA table for testing the variance between two gold labelling trials

GRID 1[a]				GRID 2			
5	3	4	1	0	2	3	6
4	2	2	2	10	2	1	2
2	2	2	2	2	1	8	4
1	1	3	8	4	1	0	5
2	3	6	3	7	2	5	9
5	0	4	2	1	0	1	3
3	6	5	4	2	4	0	3
8	0	3	3	0	4	1	4
3	0	4	10	3	0	13	1
1	5	4	7	3	2	4	0
2	6	3	4	2	20	2	1
4	2	8	5	4	2	3	1
5	1	5	2	2	2	2	3
4	5	5		1	3	2	
2	3	1		3	4	9	

[a] Gold particles/100nm^2

Source of Variation	df	SS	MS	F_s
$\bar{Y}-\bar{\bar{Y}}$ (Between groups)	1	1.24	1.24	0.1487
$Y-\bar{Y}$ (Within groups)	114	956.07	8.386	
$Y-\bar{\bar{Y}}$ (Total)	115	957.31		

$F_{0.05[1,114]} = 3.9$

$F_s < F_{0.05}$ therefore the null hypothesis is accepted. There is no significant variation between the two grids.

APPENDIX D

Raw data and nested ANOVA table testing the significance the presence of bacteria has on gold particle density.

BACTERIA ABSENT					BACTERIA PRESENT				
Wall Section					Wall Section				
1	2	3	4	5	1	2	3	4	5
5	7	5	8	10	9	5	3	7	5
3	3	5	4	15	4	2	7	4	4
4	3	5	6	9	8	4	3	2	2
5	6	5	6	12	4	8	6	5	4
9	5	9	6	5	8	7	3	4	2
10	7	6	7	6	7	5	4	8	6
7	4	7	4	8	5	7	4	6	7
6	5	10	6	7	6	7	6	9	8
4	5	6	4	4	4	8	5	4	4
3	5	6	6	6	5	3	2	2	3
6	7	8	4	3	5	4	3	4	2

Source of Variation	df	SS	MS	F_s
$\bar{Y}_A-\bar{\bar{Y}}$ (Among groups)	1	37.24	37.24	4.210
$\bar{Y}_B-\bar{Y}_A$ (Among subgroups)	8	70.76	8.845	1.979
$Y-\bar{Y}_B$ (Between counts)	100	447.28	4.47	
$Y-\bar{\bar{Y}}$ (Total)	109	555.28		

$F_{0.05[1,8]} = 5.3177$

$F_s < F_{0.05}$ therefore the null hypothesis is accepted. The presence of bacteria does not have a significant effect on the density of gold particles on hyphal walls.

$F_{0.05[8,100]} = 2.0164$

$F_s < F_{0.05}$ therefore the null hypoythesis is accepted. There is no significant variance component among wall sections.

APPENDIX E

ANOVA table for reducing sugar liberated from β-(1,3)-glucan

Source of Variation	df	SS	MS	F_s
$\overline{Y} - \overline{\overline{Y}}$ (Between Groups)	4	346.732	86.683	194.78
$Y - \overline{Y}$ (Within Groups)	10	4.45	0.445	
$Y - \overline{\overline{Y}}$ (Total)	14	351.183		

$F_{0.001[4,10]} = 5.9943$

$F_s > F_{0.001[4,10]}$ so the null hypothesis, that there is no significant varation between bacterial isolates, is rejected. The means of the five treatments are highly significantly different.

To determine which treatment produced the significant result(s), Duncan's Multiple Range Test was used. This analysis indicated that PMS164, the positive β-glucanase organism, yielded a significantly higher concentration of reducing sugar from β-(1,3)-glucan compared to the other treatments, which were in turn not significant from each other.

APPENDIX F

ANOVA table for reducing sugar liberated from α-(1,3)-glucan

Source of Variation	df	SS	MS	F_s
$\bar{Y} - \bar{\bar{Y}}$ (Between Groups)	4	0.039	0.00975	48.75
$Y - \bar{Y}$ (Within Groups)	10	0.002	0.0002	
$Y - \bar{\bar{Y}}$ (Total)	14	0.041		

$F_{0.001[4,10]} = 5.9943$

$F_s > F_{0.001[4,10]}$ so the null hypothesis, that there is no significant varation between bacterial isolates, is rejected. The means of the five treatments are highly significantly different.

To determine which treatment produced the significant result(s), Duncan's Multiple Range Test was used. This analysis indicated that PMS603, a `drippy gill' isolate yielded a significantly lower concentration of reducing sugar from α-(1,3)-glucan compared to the other treatments, which were in turn not significant from each other.

APPENDIX G

ANOVA table for reducing sugar liberated from β-(1,3)/(1,6)-glucan

Source of Variation	df	SS	MS	F_s
$\overline{Y}$ - $\overline{\overline{Y}}$ (Between Groups)	4	0.031	0.0077	1.987
Y - $\overline{Y}$ (Within Groups)	10	0.039	0.0039	
Y - $\overline{\overline{Y}}$ (Total)	14	0.07		

$F_{0.05[4,10]} = 3.478$

$F_s < F_{0.05[4,10]}$ so the null hypothesis, that there is no significant varation between bacterial isolates, is accepted. The means of the five treatments are not significantly different.

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